Investigations on Chlorophyll

Methods and Results

BY

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AND

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Authorized English Translation
From the German

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THE SCIENCE PRESS PRINTING COMPANY
LANCASTER, PA.

2896

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"Science knows no political boundaries; she recruits her conquering chieftains from all climes and races. It may be an Austrian monk, revealing the secrets of plant inheritance; or a New Hampshire farmer's boy who learns to fashion instruments of the utmost delicacy and precision; or a Serbian herdsman taking youthful lessons in communication by listening through the ground; or a Japanese devotee of medical research isolating and cultivating microorganisms. In this field all are coworkers and pride is not of race or of tradition, but of achievement in the interest of humanity."

CHARLES E. HUGHES,
Secretary of State

FOREWORD

The translation as given herein is an outgrowth of a rough translation of many of the chapters on the chloroplast pigments. The chapters were originally translated in order that the senior translator might have a better working knowledge of the physical and chemical properties of the pigments, carotin, xanthophyll, chlorophyll *a* and chlorophyll *b*. This work was begun in the Laboratory of Soil Fertility Investigations, United States Department of Agriculture, in order that methods might be developed for the very accurate determination of the amounts of the four green leaf pigments present in the leaves of plants. The methods as devised are being used in studying the effect of the various fertilizers upon plant growth. The effect of nitrates, phosphates and potash especially are being studied by the use of improved methods.

During the past six years the remaining chapters were translated and with the coöperation of the junior translator, of the Bureau of Soils, U. S. Department of Agriculture, the contents of the book have been put in its present published form.

The editing of the book has been undertaken privately because no publisher has shown his willingness to finance the publication. A canvass made of 100 members of the Botanical Society of America indicated that an edition of the book in English would be of great value to them in their scientific endeavors. Consequently, the editing was undertaken and it is hoped that the translation will prove of value to botanists and chemists who will find the book full of suggestions for many research problems.

Chemists will find the book of interest in giving suggestions for research problems which will require time and means as well as much thought for their accomplishment. Teachers, especially those in plant physiology, will find much that will interest students, especially those who are doing advanced work.

Many phases of the book will be of interest to medical men, to pharmacologists and to manufacturers.

During the past ten years about 45,000 lbs. of chlorophyll have been imported and used in the United States alone in the various industries. Commercial chlorophyll at the present time is being used: to hide color in cottonseed oil, olive oil and other seed oils; to color

various food products, stearin candles, leather, pomades, brilliantines and other cosmetic preparations; as a coloring for vaseline and to hide the color of mineral oils; to hide the ordinary yellow color of soaps and to color waxes. One form of chlorophyll is widely sold for medicinal purposes. Chlorophyll is used in many secret preparations and manufacturing concerns are attempting to use it in various new ways.

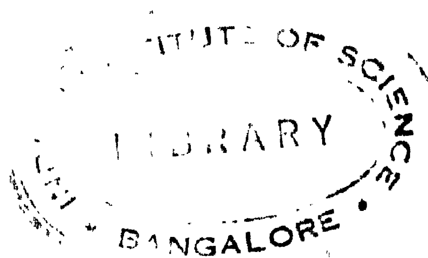
A few corrections have been made in the book. They are indicated by footnotes, marked "Trans." for translators, and the change which has been made is explained. The translators assume full responsibility for the corrections.

References are given to the works of Willstätter and his students which were unpublished at the time the book was edited, 1913. The reference to zinc and copper compounds of pheophytin is given because of its close relation to the contents of the book.

Much credit is due to Mr. P. R. Dawson, of the Laboratory of Soil Fertility Investigations, for corrections and suggestions which he has kindly made regarding the translation; Miss Fannie E. Whitney, who has so gratuitously given of her time and energy in typewriting the work, and Mrs. F. M. Schertz, who has assisted in reading proof and correcting manuscript.

The price of this book will be $4.50 and it may be purchased only from

Frank M. Schertz,
1305 Farragut St., N. W.,
Washington, D. C.
June, 1927.



PREFACE

This treatise contains unpublished investigations, made jointly with Arthur Stoll during recent years; they concern:

the isolation of chlorophyll,

the separation and the quantitative determination of all the components of leaf pigment and

the hydrolysis of chlorophyll.

In this work, new methods for the preparation and the systematic decomposition of chlorophyll were devised; then, making use of this newly acquired knowledge and of the more easily accessible materials, the earlier experiments on the transformations of chlorophyll were repeated and most of the older methods were improved.

In order to complete the work as a presentation of our knowledge of chlorophyll it is supplemented with the results of the researches which I have published with my coworkers during the last seven years in Liebig's Annalen der Chemie. It is further supplemented by some still unpublished investigations which I have made with H. J. Page on the pigments of brown algae and with M. Fischer on the relations between chlorophyll and hemin. Chlorophyll and hemin were decomposed to a common parent substance by the aid of reagents, which threw light on the essential differences in the constitution of chlorophyll and hemin.

The following communication makes chlorophyll and its derivatives easily accessible for future investigations. In imparting, with A. Stoll, the knowledge obtained, I hope to make it easier for the chemist and the physiologist to participate in research on leaf pigment.

Berlin-Dahlem, July, 1913.

RICHARD WILLSTÄTTER

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THEORETICAL INTRODUCTION

The Method.

The history of the chemical investigation of chlorophyll begins with Berzelius[1] who first undertook to isolate the pigment from leaves. Believing that it was not decomposed by either concentrated hydrochloric acid or alkalies, he treated the alcoholic extract of leaves with these strongly active reagents and therefore succeeded only in obtaining the products of radical decomposition. He decided that chlorophyll was not a resin, wax or fat but that it belonged to the dyes; he compared its coloring power with that of indigo.

The treatise of Berzelius had considerable influence upon the views and research methods of later investigators. Subsequently, G. J. Mulder,[2] F. S. Morot[3] and others attempted to isolate the pigment by means of either hydrochloric acid or alkali. F. Verdeil[4] believed that he isolated chlorophyll in a pure state when he precipitated a boiling alcoholic extract with lime water and then treated the precipitate with hydrochloric acid. To Verdeil is due the hypothesis of the relationship between the coloring matter of blood and that of leaves; according to him chlorophyll was very similar to the coloring matter of blood and likewise contained a considerable amount of iron. The iron content was assumed then for a long time, even as late as in the work of E. Schunck.[5]

E. Frémy's[6] investigations dealt with the relationship between the green and yellow pigments of leaves. As a result of the action of hydrochloric acid and ether upon the residue obtained from the evaporation of an extract of leaves, the coloring matter, according to Frémy, was distributed between a yellowish ethereal layer, containing "phyl-

[1] Ann. d. Chem. 27: 296. 1838.

[2] Journ. f. prakt. Chem. 33: 478. 1844.

[3] Ann. des Sc. Nat. (Botan.) (3) 13: 160. 1849.

[4] Compt. rend. 33: 689. 1851.

[5] Proc. Roy. Soc. 50: 308. 1891.

[6] Compt. rend. 50: 405. 1860; 61: 188. 1865; Ann. de chim. et de phys. (4) 7: 78. 1866; Compt. rend. 84: 983. 1877.

loxanthin," and a blue, acid solution of "phyllocyanin." Frémy assumed that two components of the leaf pigment were in the two layers, and that these two components formed chlorophyll by being bound together as a sort of colored fat in which phylloxanthin corresponded to glycerin and phyllocyanin to the fatty acid. Frémy afterwards altered his view and then considered chlorophyll as a mixture of these components, that is, as indifferent phylloxanthin mixed with the potassium salt of the acid phyllocyanin.

Only after a long interruption did the researches of F. Hoppe-Seyler[7] follow. These strengthened Verdeil's parallel between the blood and leaf pigments and further developed Frémy's comparison with fat to the lecithin hypothesis of chlorophyll. This work, together with the almost contemporaneous publications of A. Gautier[8] and A. Tschirch[9] and those subsequently published by E. Schunck and L. Marchlewski[10] and W. N. Hartley,[11] form a period in the investigation of chlorophyll, the results of which are of historical interest.

The methods of handling the pigment became more careful. The question whether acids and bases act destructively and what changes they produce was examined but was, to be sure, not yet correctly answered.

Chemical investigation of the pigment became more and more dependent upon the spectral-analytical method whose utility was far overrated; it did not prevent serious errors since many important changes of chlorophyll and of its derivatives exert no influence upon the absorption spectrum while, on the other hand, certain insignificant changes of constitution produce disproportionately great changes in the spectrum.

Just one result from the work of that time is lasting; recognition of a certain relationship between hemin and chlorophyll, which, of course, is much less close than was continually assumed and which became overrated in its importance.

7 Zeitschr. f. physiol. Chem. 3: 339. 1879; 4: 193. 1880; 5: 75. 1881.

8 Compt. rend. 89: 861. 1879.

9 Untersuchungen über das Chlorophyll, Berlin, 1884; Ber. d. deutsch. botan. Ges. 5: 128. 1887.

10 This work is completely reported in the three monographs by L. Marchlewski: Die Chemie des Chlorophylls, Hamburg, 1895; The article on "Chlorophyll" in Roscoe—Schorlemmer—Brühl 8: 848. 1901; Die Chemie der Chlorophylle, Braunschweig, 1909.

11 Journ. Chem. Soc. 59: 106. 1891 and 85: 1607. 1904.

Hoppe-Seyler, in the year 1879, attempted to isolate chlorophyll while avoiding energetic means, especially chemical reagents; indedendently, A. Gautier endeavored to accomplish the same. This is the correct way, as we know today, but execution of the experiment was not sucessful. Hoppe-Seyler extracted fresh grass with boiling alcohol; from the concentrated extract, by means of a series of separating and purifying operations, there resulted a crystalline preparation, chlorophyllan, of olive green color in solution. The preparations thus obtained were not impure chlorophyll. They were not chlorophyll at all. The pigment had undergone changes due to the plant acids in the extract and to heating of the alcoholic solution. It was not free from impurities. Consequently, analysis could show a phosphorus content of 1.4 per cent., and lead to the supposition that chlorophyll belonged to the lecithins. The carefully enunciated hypothesis of Hoppe-Seyler is to the present day strongly maintained by J. Stoklasa,[12] who finds potassium and phosphorus present in chlorophyll and indeed even more phosphorus than is present in lecithin.

The analysis of chlorophyllan was therefore of no value, but a reaction which Hoppe-Seyler performed with the material is of lasting importance. Upon heating the substance with alkalies at a high temperature he observed that it was converted into a purple red pigment which he called dichromatic acid. He found the dichromatic acid very unstable and therefore explained the color change caused by the hydrochloric acid as the result of a decomposition. The derivative which he obtained by means of acid treatment reminded him, in its optical properties, strikingly of hematoporphyrin, which he had obtained from hemin by means of concentrated sulphuric acid; he therefore called the chlorophyll derivative, phylloporphyrin. This is the observation with which Hoppe-Seyler experimentally established a definite connection between hemin and chlorophyll as regards molecular structure; the discovery is usually unjustly ascribed to later authors. The relationship received permanent expression in Hoppe-Seyler's nomenclature. The fact that in the analysis of dichromatic acid he overlooked its nitrogen content, does not detract from the merit of his services. The analysis was corrected by Schunck and Marchlewski, although the observations of Hoppe-Seyler on dichromatic acid were not cleared up in the course of time. According to color and fluores-

[12] Ber. d. deutsch. botan. Ges. 26: 69. 1907 and 27: 10. 1909; Mitt. d. Kalisyndikates IV, 73 and 85. 1908.

cence, dichromatic acid contains a complexly bound metal; the color change of the substance decomposable with acids does not depend simply upon the formation of a salt, as was assumed, but upon the elimination of the complexly bound metal; therefore, upon porphyrin formation. The initial material for the experiment must therefore have still contained magnesium. Of course the product was not secured in a pure or homogeneous form but phyllins were already present in the dichromatic acid while in phylloporphyrin there was present the corresponding magnesium-free substance.

The porphyrins from chlorophyll and from hemin were therefore similar to each other but not identical in all respects. Until lately no one had yet obtained identical products from the decomposition of the two pigments except by far-reaching cleavage of the pigment in reduction and oxidation. After M. Nencki and J. Zaleski[13] had obtained hemopyrrol from hemin by a reduction cleavage, using hydrogen iodide and phosphonium iodide, M. Nencki and L. Marchlewski[14] observed hemopyrrol to be produced also in the reduction of the so-called phyllocyanin-copper-acetate. From this, it is evident that the molecules of hematoporphyrin and phylloporphyrin consist of the same or closely related constituent parts. Of course, the chemical composition of hemopyrrol is much less simple than was assumed for a long time. It is not a homogeneous base; Willstätter and Asahina[15] have shown that it consists of a mixture of pyrrol homologs in which there is contained a tetra-substituted pyrrol (phyllopyrrol) but in which the tri-substituted pyrrols predominate.

Nencki[16] sought to draw biological conclusions from the relations between the porphyrins. According to him the relationship between the pigments that are important in life processes gives us an insight into the remotest past of the development of organic life and indicates a kinship between plant and animal organisms. This conclusion went far beyond its experimental foundations and was premature. That which is distinctive in the molecular structure of chlorophyll and hemin came to light only at a later date; corresponding to the quite different function of the two pigments this is much more far-reaching than the constitutional relationship.

[13] Ber. d. deutsch. chem. Ges. 34: 997. 1901.
[14] Ber. d. deutsch. chem. Ges. 34: 1687. 1901.
[15] Paper XVIII.
[16] Ber. d. deutsch. chem. Ges. 29: 2877. 1896.

After Hoppe-Seyler, chemical workers did not attempt to isolate and analyze chlorophyll. The numerous investigations of E. Schunck alone and of Schunck and L. Marchlewski[17] had to do with the cleavage products, phylloxanthin, phyllocyanin, phyllotaonin, and so forth, resulting from the action of alkalies and acids upon alcoholic leaf extracts. The genetic relationship of these compounds to chlorophyll was not ascertained and almost no data were published on their composition. The phylloxanthin, formed by acids, gave an ash which contained iron as an integral constituent; the decomposition products obtained by means of alkalies were free from mineral ingredients. Today we find that exactly the opposite is true, for chlorophyll, when treated with acids, gives ash-free substances while, on the contrary, the derivatives obtained with alkalies contain a characteristic and essential mineral ingredient.

During all the investigations of that time no chemical characteristic was established for chlorophyll by means of which it would be possible to compare the pigment when obtained from different plants; therefore, opinions regarding the identity or dissimilarity of the pigment in different plants could diverge widely. Thus, A. Gautier[18] believed that chlorophyll differed in the mono- and in the dicotyledonous plants, while A. Etard[19] in 1906 published a book, which attracted much attention, on a long series of very different chlorophylls from one plant and an unlimited number of chlorophylls from different origins.

Valuable observations of students of natural science, to whom the application of chemical methods was not familiar, exerted no influence upon the, by no means rectilinear, development of our knowledge of chlorophyll. Chemical literature did not consider the statements and suggestions of a great physicist and those of botanists, or treated them disrespectfully. As a consequence the important hints on the existence of two components of chlorophyll, which occur in optical treatises by G. G. Stokes,[20] did not fall on fruitful soil and the fascinating microscopical observations of J. Borodin[21] failed to point out the path for analysts to follow.

[17] See footnote 10 on page 2.

[18] Compt. rend. 120: 355. 1895 and also Bull. Soc. chim. (4), 5: 319. 1909.

[19] La Biochimie et les Chlorophylles, Paris, 1906.

[20] Proc. Roy. Soc. 13: 144. 1864 and Journ. Chem. Soc. 17: 304. 1864.

[21] Bot. Ztg. 40: 608. 1882.

J. Borodin noticed that under certain conditions, as when sections of leaves were moistened with alcohol and allowed to dry, crystals, which were due to chlorophyll or to a chlorophyll derivative, were formed. N. A. Monteverde[22] continued this investigation and actually had already isolated such crystals in small quantities and determined their characteristics spectroscopically. This discovery remained useless till it was repeated in a chemical laboratory. In the year 1907 Willstätter and Benz,[23] working on a large scale, obtained "crystallized chlorophyll" at a time when the analysis of chlorophyll had already been accomplished by indirect methods.

Only a few years ago, therefore, chlorophyll as such was unknown and there was no method known whereby a solution of the pigments, useful for chemical research, could be prepared from leaves. The first questions involved in analysis awaited solution; it was undecided just what elements belonged to the chlorophyll molecule. Little more was established than the fact that the decomposition products of chlorophyll belonged to the pyrrol derivatives.

To endeavor to isolate chlorophyll appeared, in the first place, hopeless because of its alterability, its chemical indifference and on account of the extreme solubility of the pigment when diluted with so many colorless and yellow, admixed substances. Without investigating chlorophyll itself, Willstätter and his collaborators deduced the characteristics of its constitution from a consideration of the derivatives that were formed upon reaction with acids and alkalies.

If an alkali hydroxide is allowed to act upon chlorophyll it is converted into salts of a chlorophyll-green color. The neutral chlorophyll has become an acid which forms water-soluble salts. In the reaction with alkalies, therefore, there has been split off hydrolytically, without any significant optical change, a component which was bound with an acid group. The mild action of acid affects another part of the molecule so that the chlorophyll color is changed to an olive green; a salt-forming group is not formed, and therefore saponification is avoided. Hence, by acid cleavage one succeeds in sparing and in finding in the products of cleavage those components of chlorophyll that are split off by alkalies and conversely the alkali derivatives of the pigment must show another characteristic atomic group which is extremely easily

[22] Act. Horti Petropol, 13, no. 123, 1893.

[23] Paper VI.

destroyed by acids. As a consequence of this guiding conception[24] it was possible, before chlorophyll itself was known, to combine its properties from the analysis of the decomposition products that are formed by acids and alkalies, and so perfectly was this done that when the preparation of the natural pigment in the pure state was finally successful nothing new was learned from the analysis of it.

The chlorophyll-green carboxylic acids, the chlorophyllins, that are formed by the alkaline hydrolysis of alcoholic leaf extracts, are, it is true, very unstable, but they could be separated in a somewhat pure state from their mixture with the other products of the saponification. They were extracted from their ethereal solutions by di-sodium phosphate and released again by mono-sodium phosphate. On analysis[25] they proved to be magnesium compounds in which the magnesium is complexly bound; not in a state capable of electrolytic dissociation as in magnesium salts, but in a peculiar mode of union and stable relation in which the ionic reactions of the metal are lacking. The magnesium-containing group is, to be sure, very sensitive to acids, but it is uncommonly stable toward alkalies so that during radical changes of the molecule, in which even the carboxyl groups are successively split off, the magnesium-containing group remains intact. As a consequence, proof of the presence of magnesium and of its manner of combination was confirmed by decomposition[26] of the chlorophyllins by heating (to 240°) with concentrated alcoholic alkalies; when this was done there was formed a series of beautifully colored, well-crystallizing, intensively fluorescent decomposition products, the so-called phyllins, which contain three, two, or finally only one, carboxyl group. All these compounds, a number of which, such as glauco- and rhodophyllin, have received their names from their beautiful blue and red colors and of which we later describe a dozen different ones, are magnesium-containing carboxylic acids. In the investigations that are communicated here the systematic decomposition is continued down to the carboxyl-free parent substance, etiophyllin, whose composition is expressed by the fomula



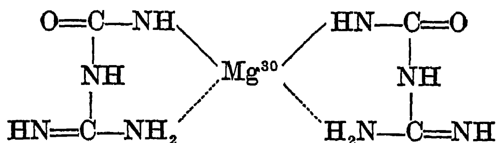
$$C_{31}H_{34}N_4Mg.$$

The ash content, in consequence of the diminution of the molecule, has risen here to 8 per cent of magnesium oxide.

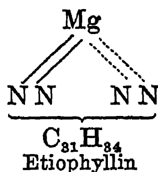
[24] Paper III.
[25] Paper II.
[26] Papers V, VIII, XXII.

All the phyllins contain one atom of magnesium to four atoms of nitrogen. The oxygen atoms, namely, the carboxyl groups, take no part in the formation of the metal complex; only the nitrogen-containing groups of the molecule are available for binding the magnesium with primary and secondary valences.

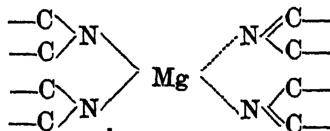
This conception, in harmony with the observations of A. Werner[27] on the constitution of complex metallic compounds and in analogy with the metallic derivatives of the acid imides, biuret and dicyandiamidine, investigated by H. Ley[28] and L. Tschugaeff,[29] is, for example, expressed by the formula:



in which the affinity of the magnesium is not exhausted in the two valences which are linked to the nitrogen of the two pyrrol nuclei; it is also linked by partial valences to two other pyrrol nitrogens to form the complex,



or in more detail:



Under the influence of acids all phyllins lose magnesium and are transformed into polybasic and monobasic carboxylic acids which display characteristic basic, in addition to acid, properties. Since they

[27] Neuere Anschauungen auf dem Gebiete der anorganischen Chemie, 2nd Edition, Braunschweig, 1909.

[28] Ber. d. deutsch. chem. Ges. 40: 705. 1907.

[29] Ber. d. deutsch. chem. Ges. 40: 1973. 1907.

[30] Trans. Changed to Mg from Me as given by Willstätter.

form a natural group with phylloporphyrin which was investigated by Hoppe-Seyler, Tschirch, Schunck and Marchlewski, even though not isolated in a pure state, they are designated as porphyrins, each with the prefix of the corresponding phyllin.

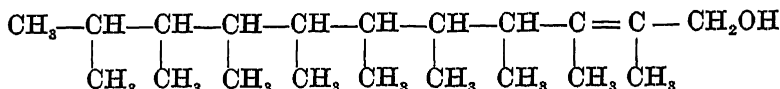
The behavior, toward acids, of the phyllins that are formed by decomposition enlightens us regarding the action of acids upon a solution of chlorophyll. A visible change takes place; the color changes to olive and the fluorescence becomes weaker. The magnesium-free derivative of chlorophyll, which has been called pheophytin, has become a very easily obtainable product as a result of the discovery that it precipitates almost completely and in an immediately pure state upon the careful treatment of an alcoholic solution of crude chlorophyll with oxalic acid. As a result, a method which permits chlorophyll to be separated in the form of a pure derivative replaces the bothersome and but slightly productive procedure by means of which Hoppe-Seyler obtained chlorophyllan as a product of the unintentional decomposition of the pigment through the influence of plant acids. The method permits pheophytin to be obtained in kilogram lots in the laboratory; the meal of dried nettle leaves is a very satisfactory material for extraction.

Pheophytin does not have any ash and is free from colorless and yellow impurities; it is a waxy substance without acid properties and is weakly basic in nature.

It differs considerably from chlorophyll in the color of its solutions. By introducing into its molecule a metal, which enters into complex combination, this cleavage product is immediately made similar to chlorophyll again. Many metals, for example, copper and zinc, are introduced very easily by the action of their acetates upon an alcoholic solution of pheophytin. It was much more difficult to reintroduce magnesium because of the sensitiveness of the magnesium complex toward acids. This was finally accomplished by means of Grignard's reagent, for example, by the action of magnesiummethyl iodide upon pheophytin or upon the porphyrins. A second method, the action of magnesium oxide and alkali with the application of heat, was not applicable for the re-formation of chlorophyll because of the instability of chlorophyll in the presence of alkalies, but was very suitable for the formation of phyllins from the porphyrins.

Pheophytin, on saponification with alkalies, behaves like a wax and produces, besides high molecular, nitrogen-containing acids with 34

carbon atoms, a nitrogen-free alcohol with 20 carbon atoms, which Willstätter and Hocheder[31] discovered and called phytol. It corresponds to the formula $C_{20}H_{39}OH$ and is an unsaturated primary alcohol with an open chain of carbon atoms. Investigations concerning its decomposition have made it probable that its carbon skeleton has many side chains; the following constitutional formula—of course hypothetical in its details—may give a provisional picture of its structure:



It is not improbable that a relationship exists between isoprene, the well-known building stone of the terpenes and caoutchouc, and this alcoholic component of chlorophyll. The chlorophyll derivative that is formed by acid has consequently yielded the information which was sought concerning the chief transformation which alkalies produce upon the molecule of chlorophyll; they saponify the phytol ester group. The action, however, is not limited to this group, for pheophytin, and hence chlorophyll, contains, in addition, a $COOCH_3$ group, which is decomposed in the second step of hydrolysis.

In addition to the alteration of the two ester groups, there takes place as a result of the action of alkali a further, peculiar transformation, which is betrayed by a remarkable color change, the so-called brown phase. The solution of pheophytin, as well as that of chlorophyll, becomes intensively brown when mixed with methyl alcoholic potash; then, in a few moments, the original color of the liquid returns. On standing in alcoholic solution, chlorophyll easily undergoes a change in which it loses this characteristic of the brown phase.

Phytol constantly appears as a component of chlorophyll of which it makes up a third of the molecule. This knowledge was obtained in an indirect manner by working up different plants. Pheophytin preparations of different origin at first showed considerable fluctuations. The phytol content never exceeded 33 per cent, was frequently less than this and in many cases sank to zero. Now, it has been found that just those plants that contained chlorophyll that was poor in phytol proved to be excellent material for the isolation of chlorophyll in the form of very beautiful crystals. These crystals were identical with

[31] Paper III.

those which the Russian botanist Borodin had already discovered in microscopical leaf sections in 1881.

Monteverde, in his spectroscopical investigation, had declared that this crystallized chlorophyll was the natural pigment and regarded amorphous chlorophyll as a decomposition product. This view could no longer be maintained. Crystallized chlorophyll is phytol-free and is a derivative of the natural, phytol-containing pigment, which owes its easy solubility and more wax-like nature to its content of high molecular alcohol.

The production of crystallized chlorophyll on a preparative scale and its investigation was of special value till it was possible to separate the natural dyestuff itself in a pure condition.

The formation of the crystallized compound and the corresponding deficiency of phytol has recently been explained by our observation that in the case of rapid extraction of many leaves the phytol content is normal, but that in the case of slow extraction, *i.e.*, when the extract remains in contact with the meal of dried leaves for some time, it is too low. Chlorophyll in the green plant parts is accompanied by an esterase enzyme, chlorophyllase. This is not inactive in alcoholic media, as is generally assumed to be the case with enzymes, but it causes displacement of the phytol by the alcohol and thus effects an alcoholysis of the chlorophyll. The enzyme is very widely distributed but the amount present varies within wide limits. After the dynamics of the enzyme reaction had been sufficiently studied, application of the action of chlorophyllase was made on an extensive scale for preparative purposes. The formation of "crystallized chlorophyll" is no longer a matter of chance. Almost all the chlorophyll can be separated from fresh as well as from dried leaves either in the form of the methyl or ethyl compound (methyl or ethyl chlorophyllide) or, by hydrolysis, in the form of the corresponding free carboxylic acid, the chlorophyllide.

Conversely, partial synthesis of chlorophyll from the two components can also be carried out, namely, from chlorophyllide with the alcohol, phytol, by esterification under the catalytic influence of chlorophyllase. The usual methods of ester formation should not be applied in this case on account of the sensitiveness of chlorophyll.

Since pheophytin is the most suitable form of chlorophyll for investigational purposes as well as for the comparison of the leaf pigment from different plants, a description of chlorophyll presupposes,

besides determinations of the magnesium and phytol, first of all a knowledge of the nitrogen-containing carboxylic acids which appear, in addition to the phytol, upon the saponification of pheophytin. Pheophytin is, in truth, a pure chlorophyll substance although it is not a homogeneous compound; its acid component is composed of substances which differ in basic properties and color.

In the beginning, the investigation led to a great number of such decomposition products, which form two groups: those of one, the phytochlorins, are olive green in indifferent solution, those of the other, the phytorhodins, are beautifully red. On account of their great number, the single compounds have been designated by the group names with letters attached to them.

It would hardly have been possible to have shed light on a mixture of such complicated composition as was encountered during the first transformations of chlorophyll if the basic nature of the decomposition products had not placed in our hands as a result of their unusual differentiation a never-failing method for the separation and the determination of the chlorophyll derivatives. This method, which was devised by Willstätter and Mieg,[32] depends upon the different distribution of these dyestuffs between ether and dilute hydrochloric acid. A chlorophyll derivative is characterized by the concentration of the acid (hydrochloric acid number) necessary to extract it from ether. The ratio in which these bases are distributed between ether and dilute acids varies exceedingly with the concentration of acid used. The compounds in a mixture are, therefore, separated by fractionating their ethereal solution with hydrochloric acid of different percentage concentration. By this means a great number of pure phytochlorins and phytorhodins have been successfully prepared from pheophytin.

The appearance of the complicated mixtures was, however, merely a result of certain transformations that the chlorophyll, which is easily alterable in alcoholic solution, underwent under the experimental conditions; as, for example, during too slow extraction or too slow precipitation with acid. The preliminary treatment of the plant material, and particularly its extraction and the treatment of the extract with acid, had to be improved and to be made more uniform. By looking for the causes of the changes appearing in the solutions and by learning to avoid them, the differences between the preparations became less frequent and more insignificant and we finally suc-

[32] Paper I.

ceeded in obtaining two, and only two well-crystallizing and characteristic, cleavage products from pheophytin:

Phytochlorin *e*, of the composition $C_{34}H_{34}O_5N_4$ and
Phytorhodin *g*, of the composition $C_{34}H_{34}O_7N_4$.

Phytochlorin *e* is a tricarboxylic acid with two free carboxyl groups and one bound as a lactam. Phytorhodin *g* is a tetracarboxylic acid, which has only two or three of its carboxyl groups in the free condition.

The joint appearance of a green and a red decomposition product upon consecutive hydrolysis of chlorophyll with acid and alkali opened up an important question. Is it caused by the decomposition of a larger molecule into two fragments? As an argument against this we find that the molecular weight of pheophytin is of similar magnitude to that of phytochlorin and phytorhodin. Also, it is conceivable that one of the cleavage products is an earlier and the other a subsequent state of the decomposition. Phytochlorin and phytorhodin, however, cannot be converted, the one into the other, and, also, they are formed in quite definite proportions by weight. It may be concluded from the formation of phytochlorin *e* and phytorhodin *g* that it is much more probable that pheophytin, and consequently chlorophyll, is a mixture of two components, one of which, on decomposition, produces phytochlorin *e* and the other phytorhodin *g*.

On the strength of this assumption we endeavored to effect a separation of the components of the mixture by physical and chemical means.

One method, which is used for chlorophyll solutions, crystallized chlorophyll or pheophytin, consists in a displacement of the given component ratio by unequal distribution of the dyestuff mixture among several non-miscible solvents, for example, between aqueous methyl alcohol and petroleum ether; or, in the case of the difficultly soluble phytol-free compounds, methyl alcohol and ether-petroleum ether. By numerous repetitions of the operation this displacement of the component ratio can be increased and so utilized that the two components of the magnesium-free or magnesium-containing dyestuffs are finally obtained in a pure state.

The other method, which is, of course, applicable to magnesium-free compounds only, consists in fractionating with hydrochloric acid according to the method of Willstätter and Mieg. Pheophytin is such a weak base and its phytol ester group is so sensitive toward hydrolytic

agents that this separation offered special difficulties and was not easily carried out. It was successful, however, and confirmed the results of the method of separation by non-miscible solvents.

With the establishment of these facts, the supposition which the English physicist Stokes[33] expressed, though unfortunately in only a few short words, in 1864, was confirmed. Stokes recognized by means of the spectroscope that chlorophyll was a mixture and endeavored to separate it by distribution between alcohol and carbon disulphide, thus laying the foundation of the method of separation by non-miscible solvents, which was subsequently perfected by H. C. Sorby[34] and G. Kraus.[35] It was used mostly to demonstrate that yellow pigments accompany the green dyestuff.

More recently the botanist M. Tswett,[36] of Warsaw, has confirmed Stokes' view in an odd way, *i.e.*, by obtaining a separation of the natural pigment on an analytical scale by means of its fractional adsorption from its solutions. Although it had heretofore been absolutely impossible to ascertain whether alterations and, perhaps, cleavages of the pigment had first occurred during the operations involved in the separation, the splitting of the pigment into its typical cleavage products now makes proof of an unaltered pigment nucleus possible. A decision can now be made for the first time between the for a long time almost forgotten statements of Stokes and the many opposing ideas, such as the statements of Étard about the unlimited variability of chlorophyll, since the chemical characteristics of the green leaf pigment have been sufficiently well established. The chemical characterization of chlorophyll was thus a prerequisite for the isolation of the pigment in an undamaged and pure state as a mixture of its components (*a* and *b*) and for the confirmation, by means of its analysis, of the deductions which had been previously drawn from the investigation of its derivatives.

Chlorophyll has the following characteristics, which have been established by systematic decomposition with alkalies and acids.

It contains 4.5 per cent of ash which is pure magnesia.

Chlorophyll and pheophytin, when saponified by boiling for a short time with methyl alcoholic potash, yield the normal mixture of phyto-

[33] l. c.

[34] Proc. Roy. Soc. 21: 442. 1873.

[35] Zur Kenntnis der Chlorophyllfarbstoffe und ihrer Verwandten. Stuttgart, 1872.

[36] Ber. d. deutsch. botan. Ges. 24: 316, 385. 1906.

chlorin *e* and phytorhodin *g*. Besides, a third of the weight of the molecule is liberated as phytol, a colorless oil. The dyestuff no longer contains any yellow pigments, for they would be observed by extracting with ether after the saponification.

If the action of the alkali is carefully carried out at room temperature, the brown phase must appear; with the pure *a* component of chlorophyll the color change is to yellow, while with chlorophyll *b* it is to red.

Finally, the spectroscopical comparison of chlorophyll with a fresh leaf extract should be mentioned; in the initial stages of the decomposition of chlorophyll, absorption in the green region of the spectrum increases strikingly.

The solution of problems of preparation has been furthered by new methods of extraction which will be published in this treatise.

Dried, pulverized leaves were used as material when working on a large scale. It turned out to be the case that considerable water in the solvent materially hastens the extraction of the total dyestuff of the leaf. The pigments occur in a colloidal condition in the chloroplasts even after the leaves have been dried, and are in this condition difficultly soluble. They are separated as floccules by a solvent that dissolves the salts from the leaf substance, and are thereby rendered more readily soluble. In order to isolate the chlorophyll, it is transferred from the extracting solvents to petroleum ether. The great quantity of the admixtures, which accompanies chlorophyll into the aqueous solvent and facilitates its extraction, has a far less unfavorable effect upon the degree of purity of the petroleum ether solution than the admixtures that accompany chlorophyll in alcohol or acetone extracts which contain only a small percentage of water.

The isolation of chlorophyll, which Willstätter and Hug[37] were successful in bringing about two years ago (1911), depends upon the colorimetric determination of the purity of its solutions and upon a systematic increase of the purity by methods of separation by means of non-miscible solvents. Distribution among several solvents of the materials that are contained in the extracts is here utilized in a special manner to separate the yellow and also the colorless substances that accompany the chlorophyll. By means of these operations solutions containing about 70 per cent of chlorophyll are obtained from extracts which, on account of the great quantity of colorless accompanying

[37] Paper XV.

substances, contain only 8–16 per cent of chlorophyll. Then, at last, an unexpected observation aids in the solution of the problem. When the chlorophyll has reached a certain degree of purity it is still easily soluble in petroleum ether that contains alcohol but, surprisingly, it is no longer soluble in pure petroleum ether. If the ethyl or methyl alcohol is removed by washing, the chlorophyll separates and can be purified by reprecipitation from an ethereal solution by means of petroleum ether.

This procedure was at first laborious and the yield was small. Using our new method of extraction with aqueous acetone, however, the method was so improved that pure chlorophyll can now be isolated without much trouble from a few kilograms of nettle leaves in a few hours and with a yield of about 80 per cent of the total chlorophyll content, or about 6.5 g. from a kilogram of dry leaves.

The procedure can also be carried out with fresh leaves, and, as in the whole course of the work, there is in the production of chlorophyll preparations or in their properties no difference between freshly plucked and dried leaves. In a lecture hour a quarter of a gram of pure chlorophyll can be isolated from a quarter of a kilogram of fresh nettle leaves. With more time, 4 g. of chlorophyll may be obtained in one operation from 2.5 kg. of fresh leaves, that is, about four-fifths of their chlorophyll content.

We distinguish between methods for the preparation of chlorophyll in the pure state and those for obtaining crude products which are suitable as initial material. Such crude products, containing 90–95 per cent of chlorophyll, are easily obtained by extraction with aqueous acetone.

Today, therefore, the leaf dyestuff can be isolated at least as easily as any other plant constituent, such as an alkaloid or a sugar.

The separation of the yellow accompanying substances, which are exceedingly widely distributed in plants, and which are associated with chlorophyll in the chloroplasts, is an important problem in the preparation of pure chlorophyll. The joint occurrence of the yellow dyestuffs with the green indicates a significant physiological role for these carotinoids and has induced us to prepare them also in a pure state and to analyze them. They become by-products in the production of chlorophyll preparations.

There are present in every green leaf two easily crystallizable nitrogen-free pigments which have many properties in common but which differ in their behavior toward solvents and which have been

distinguished by J. Borodin[38] and other botanists by this means. One of these pigments is, as was already shown to be probable by A. Arnaud,[39] identical with the carotin of carrots which has been known for a long time. Analyses by Willstätter and Mieg[40] have shown that it is an unsaturated hydrocarbon of the formula $C_{40}H_{56}$. Its companion, xanthophyll, was as yet unknown in substance, in spite of the fact that there is always a greater quantity of it in the leaf. It is to be considered as an oxide of carotin because of its composition $C_{40}H_{56}O_2$. The hydrocarbon is quite soluble in petroleum ether while the oxygen compound, on the other hand, is easily soluble in alcohol only. The two yellow pigments show a great affinity for oxygen, which they absorb greedily, especially in solution.

A third carotinoid, fucoxanthin, whose isolation and properties are described later, is found in brown algae; wherever it is present the amount of the other two carotinoids is reduced. The formula of fucoxanthin is $C_{40}H_{54}O_6$; in its chemical relations it is similar to carotin and xanthophyll but it is distinguished from them by the pronounced basic properties of its oxygen atoms and by the formation of a characteristic blue hydrochloride.

With the chemical characterization of chlorophyll and its accompanying pigments the preliminary conditions are fulfilled for ascertaining the relative quantities of all the components of the pigment of leaves and for comparing the green pigments in the different classes of plants. Over 200 plants, from numerous classes of Cryptogams and Phanerogams, furnished material for this comparison with respect to the chemical characteristics of chlorophyll. Our method consisted in testing the pheophytin, which had been separated by quick extraction of the leaves and quick precipitation with acid, as to its phytol content as well as to its basic cleavage products, phytochlorin *e* and phytorhodin *g*, and, supplementarily, the decomposition of the chlorophyllin alkali salts to crystalline rhodophyllin which has an ash content of 7.02 per cent magnesium oxide.

The result shows that chlorophyll is identical in all the plants investigated. Only a single leaf-green which consists of the two components, *a* and *b*, of chlorophyll is found. A great regularity is also observed in the quantitative relation between these; chlorophyll *a* pre-

[38] Mélanges biologiques tirés du Bull. de l'Acad. Impér. de St. Pétersbourg 11: 512. 1883.

[39] Compt. rend. 102: 1119. 1886; 104: 1293. 1887; Bull. soc. chim. 48: 64. 1887.

[40] Paper IV.

dominates to the extent of almost three molecules of chlorophyll *a* to one of chlorophyll *b*.

The Pheophyceae, in which in addition to chlorophyll *a* only an exceedingly small quantity of the *b* component is present, are an exception to this quantitative relation.

This investigation of the component ratio, a detailed account of which is published in Chapter IV, concerns the two green as well as the two yellow pigments of the chloroplasts. The former were separated in the form of their cleavage products, phytochlorin *e* and phytorhodin *g*, by fractionating with hydrochloric acid, and were determined colorimetrically. The carotinoids, which were separated from the green pigment after its saponification with alkali, were, on the other hand, fractionated by means of their different distribution between petroleum ether and dilute methyl alcohol in order to determine them likewise quantitatively by a colorimetric procedure. The analyses showed, as yet, no significant dependence of the component ratio upon external factors such as the season, the time of day and illumination, and no great difference between different plant species; under extreme conditions of life deviations from the average ratio were not more than 30 per cent.

The molecular proportion of the green to the yellow pigments is also approximately constant, namely 3:1, and the ratio of carotin to xanthophyll, with insignificant fluctuations in the leaves exposed to direct sunlight, amounts to 0.6:1.

The amounts and the quantity ratios of the pigments may be illustrated by the results of an analysis.

In 1 kg. of dry elder leaves (corresponding to 4 kg. of fresh leaves) there are contained:

8.48 g. of chlorophyll, that is,
6.22 g. of chlorophyll *a*, 2.26 g. of chlorophyll *b*.
1.48 g. of carotinoids, that is,
0.55 g. of carotin and 0.93 g. of xanthophyll.

These quantities correspond to the following molecular proportions:

For 1 molecule of chlorophyll $(a+b)$ there occurs 0.35 molecule of carotinoids.

For 1 molecule of chlorophyll *a* there occurs 0.36 molecule of chlorophyll *b*.

For 1 molecule of carotin there occurs 1.61 molecules of xanthophyll.

As long as the mixture of the chlorophyll components served as initial raw material for systematic decomposition there was no reaction which would lead to a homogeneous product. In fact, a homogeneous product can not be easily produced even from a pure component by any transformation. Since the complicated molecule offers numerous points of attack, much more complicated mixtures of reaction products result from the simultaneous transformation of the two chlorophyll components; only in the later stages of decomposition can the two components, which are related in structure, be successfully transformed under exactly controlled conditions into the same derivatives, pyrrophyllin and phyllophyllin.

Observation of the reactions is consequently simplified if homogeneous initial materials are used.

Chlorophyll consists of the blue-green component *a*, which forms, in solution, an olive-green pheophytin, and the yellowish-green component *b*, whose magnesium-free derivative is red-brown in neutral solutions. When distributed between methyl alcohol and petroleum ether more of the *b* component passes into the oxygen-containing solvent; analysis shows that the *b* component is richer in oxygen. The relations between the two components as to composition are made somewhat hazy by the fact that numerous compounds of this group are inclined to form hydrates and appear sometimes with water and sometimes without water, being frequently combined with a half molecule.

The material for analysis consists of magnesium compounds and their magnesium-free derivatives. The simpler phytol-free compounds, such as the methyl derivatives, as well as the free chlorophyllides and the pheophorbides, are more useful than are the phytol compounds for this purpose; the acids that are formed by hydrolysis of the phytol ester contain a carboxyl, designated by α, and are named pheophorbides. Besides, the analysis of phytochlorin *e* and phytorhodin *g* is important in acquiring a knowledge of the difference between the two series.

From this it has been found that the two chlorophyll components agree not only in their magnesium and phytol content but that they are also very similar in the composition of the basic nucleus; this relationship is repeated in all the parallel steps of decomposition. From the analyses it is concluded that the difference between the *a* and the *b* series probably consists in a molecule of oxygen, two hydrogen atoms

of chlorophyll *a* being replaced by one oxygen atom in chlorophyll *b*, in accordance with the formulae:


Chlorophyll *a*, $C_{55}H_{72}O_5N_4Mg$,


Chlorophyll *b*, $C_{55}H_{70}O_6N_4Mg$.

This assumption has by no means been proved; the assumption is used provisionally in order to render the relations between the two components understandable, not forgetting that further investigations are needed to fix and strengthen our explanation; this is an object of future work.

Neither of the components has as yet been oxidized or reduced to the other; yet, many reactions are known which indicate a simple difference in the degree of oxidation of the two series, for instance, the action of Grignard's magnesium compounds upon phytorhodin whereby, as a result of the addition of hydrocarbons, compounds of the *a* series are formed.

The relationship between the two components, as regards composition and constitution, is significant in explaining the chemical function of chlorophyll. Analytical investigation gives some clues to a conception of it on the assumption that the *b* component is an oxidation product of the *a*.

The rôle of the magnesium may be considered as similar to that in the organo-magnesium compounds discovered by Barbier and Grignard, which have attained such great importance in organic synthesis because of their facility of reaction. A comparison was drawn between chlorophyll and Grignard's compounds even in our first publication[41] on the analysis of chlorophyll. The parallel[42] appeared to be inexact and met opposition because it disregarded the difference between the linking of the metal to carbon in the ordinary organic magnesium compounds and substitution at the nitrogen in chlorophyll. This difference, however, is not considered as either a pronounced or characteristic one.

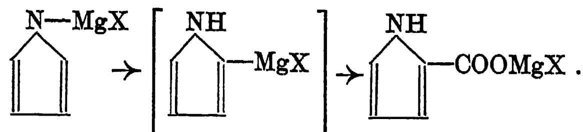
Since our publication, B. Oddo[43] has conducted important investigations on pyrrolmagnesium iodide which reacts with carbon dioxide and acid chlorides with the formation of α-substituted pyrrols, α-carbopyr-

41 Ann. d. Chem. 350: 65. 1906.

42 See also V. Grignard, Bull. soc. chim. (4) No. 11, 13. Appendix 1913.

43 Gazz. chim. ital. 39. I 649. 1909 and Ber. d. deutsch. chem. Ges. 43: 1012. 1910.

rolic acid and alkylpyrrylketones. The N-magnesium derivative is probably formed at first, and this either changes further into the α-magnesium compound or reacts as such. For instance



The pyrrolmagnesium derivatives—comparable to sodium aceto-acetic ester—behaved, therefore, similarly to any Grignard substance having the metal linked to carbon.

Chlorophyll may be considered as belonging to the same class of organic magnesium compounds and the drawing of a sharp line between magnesiumphenyl iodide, pyrrolmagnesium iodide and chlorophyll does not appear justifiable. Chlorophyll is only distinguished from the ordinary organo-magnesium compounds, in consequence of the additionally complex linking of the metal, by a greater stability of the magnesium toward water.

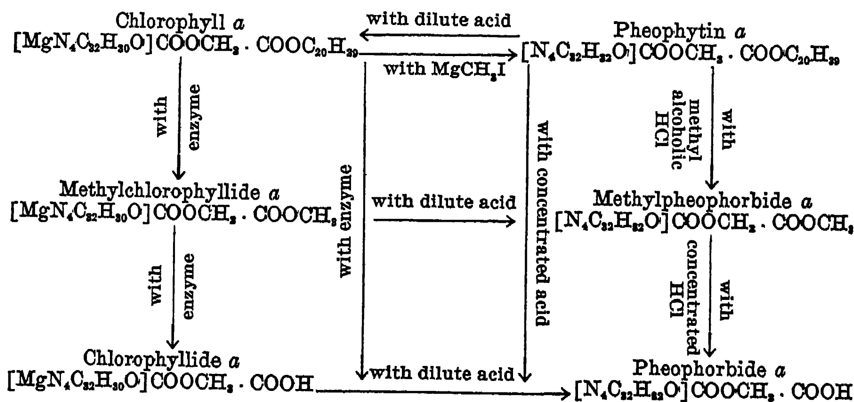
This comparison does not require that the pigment, in the process of assimilation, incorporate carbon dioxide into its molecule; this can be prevented by substitution in the magnesium-bearing pyrrol nucleus. The function of chlorophyll may rather be imagined thus: carbon dioxide is attracted by the affinity of the magnesium compounds and its reduction is then brought about by chlorophyll component *a* in a process which uses up the absorbed light energy. Chlorophyll *a* is oxidized to chlorophyll *b* in this process and this is again transformed into chlorophyll *a* by the splitting off of oxygen; a state of equilibrium appears between the two components.

It is possible that either this giving up of the oxygen by the component *b* proceeds directly, or that the yellow pigments, carotin and xanthophyll, take part in the transformation back to chlorophyll *a*. Since the carotinoids constantly accompany the green pigments in the chloroplasts it is probable that they are functionally related. Perhaps their purpose is to regulate the ratio of the chlorophyll components somewhat like this: carotin withdraws oxygen from chlorophyll *b*, and the oxygen is set free, from the xanthophyll that is thus formed, by the action of an enzyme.

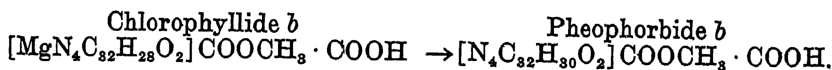
The method for the determination of the four components of the leaf pigment assists in investigating more fully the process involved in the assimilation of carbonic acid.

Questions of Constitution.

The first changes of chlorophyll, which will be explained by means of formulae in the case of component *a*, which is used illustratively, concern the *a* carboxyl that is bound with phytol. To the older methods of systematic decomposition with acid and alkali there has been added a third method—enzymatic cleavage by means of chlorophyllase—by means of which the simple alphyl chlorophyllides are formed in alcoholic solutions and the free chlorophyllides in aqueous media. These compounds are obtainable in no other way. Upon elimination of the magnesium with acid they produce alphyl pheophorbides, which can be obtained from pheophytin by alcoholysis with hydrochloric acid and methyl or ethyl alcohol, and the free pheophorbides, which are obtained from pheophytin by hydrolysis with concentrated hydrochloric acid.



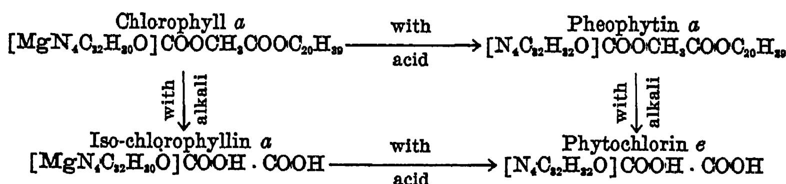
In the *b* series the first changes run an analogous course and give the following stages of decomposition:



$$\underset{\text{Chlorophyllide } b}{[MgN_4C_{32}H_{28}O_2]COOCH_3 \cdot COOH} \rightarrow \underset{\text{Pheophorbide } b}{[N_4C_{32}H_{30}O_2]COOCH_3 \cdot COOH.}$$

The effect of alkalies is more complicated. They first attack that place which remains unaffected by the other procedures; namely, the third, latent carboxyl which is present in the form of a lactam group. From each chlorophyll component there is formed, according to the conditions of the alkaline saponification, two alkali salts which are obtained in a homogeneous state, one of which fluoresces like chloro-

phyll while the other lacks fluorescence. The alkali compounds, potassium chlorophyllins *a* and *b*, which are obtained chiefly by mild saponification in the cold, are converted, when the magnesium is removed, into weakly basic phytochlorins and phytorhodins that are of relatively small importance in the characterization and systematic decomposition of the chlorophyll components. The other chlorophyllins, which were not obtained till later and were consequently designated as iso-compounds, are formed by quick saponification with heat and are the complex magnesium compounds of phytochlorin *e* and phytorhodin *g*; they are converted into these most important cleavage products of chlorophyll by acidification. Thus, the same derivatives have been obtained, although only recently, by successive treatment with alkali and then with acid as by the reversed order of the process.



But the relation between chlorophyll and iso-chlorophyllin is not as simple as this scheme permits us to expect; the alkalies not only saponify two ester groups but they produce, first and foremost, a transformation which is recognized by the appearance of the "brown phase."

A theory for this brown phase can alone give a key to an explanation of the first stages of the decomposition, the alteration of chlorophyll in its solutions and the formation of different series of chlorophyllins.

Let us bear in mind that when alkalies act upon chlorophyll and the chlorophyllides the green color at first changes to an intensive brown, the *a* component changes to a yellowish brown and the *b* to a red, and then in a few minutes the original color of chlorophyll returns in the alkaline media. The reaction creates the appearance of a complete decomposition and a reformation of the chlorophyll. It may, of course, be understood as follows: a group, forming an essential part of the chromophoric complex, is changed by hydrolysis and a new similar group is formed in its place.

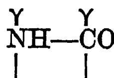
We attempt to explain this behavior of chlorophyll as a "relactamization", as the opening of an existing lactam ring and the closing of a new ring, similar but stable in alkali.

The phenomenon is not confined to the magnesium compounds. The pheophytins and the pheophorbides give a similar brown phase with alkali; this is the only instance in which the magnesium-containing and the magnesium-free compounds assume the same appearance. In consequence, we consider the complex state of the magnesium as non-existent during the brown phase. The phytochlorins and phytorhodins are formed from the pheophorbides during this phase; consequently a carboxyl must be bound lactam-like in these cleavage products also. Many observations harmonize with this theory.

Not only can the lactam group of chlorophyll open, but we are able to bring about a reformation of the original group. This occurs when an ethereal chlorophyll solution is mixed with methyl alcoholic potash and then with water. The substance first passes quantitatively into the alkaline layer with a brown color, then, as a result of the hydrolytic dissociation of the potassium compound, it returns intact to the ether; it has not acquired any acid properties and again gives the brown phase. The original lactam ring is, therefore, not very stable, yet it forms very easily. Other lactam groupings are formed more slowly but are more stable; relactamized chlorophyll is not split up by alkalies any more.

The formation of a new lactam ring takes place in several ways. The different series of chlorophyllins and the occurrence of the weakly basic phytochlorins and phytorhodins, which are so difficult to avoid, are explained by this fact.

The original group may be expressed:

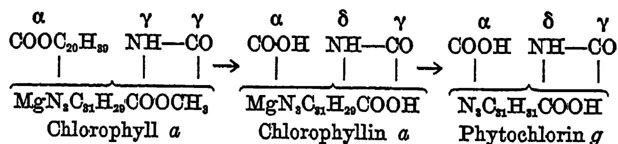


It may relactamize in such a way that the carboxyl, γ, enters into a different combination with the same nitrogen atom, or with another nitrogen group, which may be called δ, or the relactamization occurs in such a way that another carboxyl, namely *α*, unites, for example, with the nitrogen atom, γ.

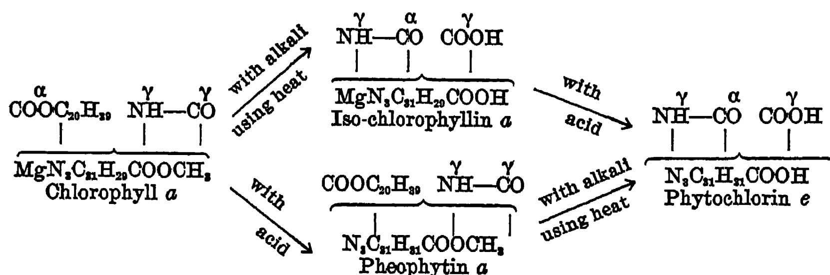
Two courses of chlorophyllin formation are—for the sake of simplicity we will use chlorophyll *a* as an example—disclosed as follows:

1. On saponification in the cold the lactam group is first opened, then the γ carboxyl, which has been liberated, combines for the greater part with the δ nitrogen atom, and this happens even when the *α*

carboxyl is already free, namely, when the acid chlorophyllides are saponified:



2. In saponification with the application of heat, the α carboxyl, which is liberated from the α ester group or which is already free, combines with the γ nitrogen atom of the original lactam group. It is probable that, of several possible transformations, this one is especially accelerated in cases of energetic saponification, for example, by the use of very concentrated alkali and the application of heat, and may be quantitatively carried to completion by this means.

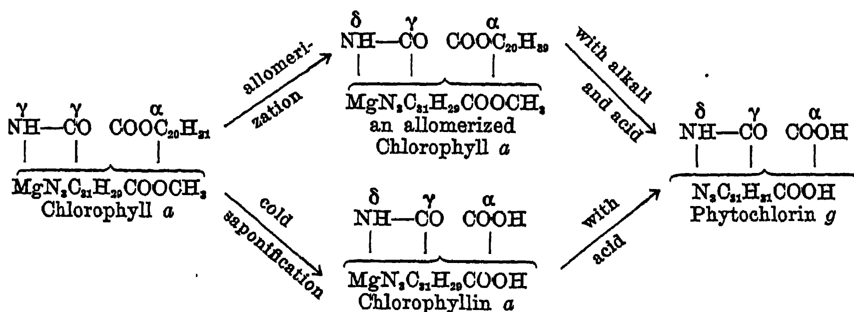


The lactam theory of the brown phase, although still uncertain as to its details and requiring further development, also explains in a satisfactory manner the changes which chlorophyll, the alkylchlorophyllides in general, and the free chlorophyllides especially easily, undergo on standing in alcoholic and other solutions. These changes are termed allomerization and the products that are formed are called allomeric chlorophyll derivatives. Allomerization may be recognized by the fact that the compounds lose their power of crystallization and, in place of the normal cleavage products, yield feebly basic phytochlorins and phytorhodins. Naturally, an allomeric derivative no longer gives the brown phase.

The phenomenon depends upon a change in lactam groups, namely, upon the fact that the original lactam group is opened by means of alcohol. This opening would produce a state of equilibrium between the lactam form and the open form if an increasing amount of the

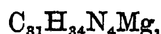
original form were not removed from the equilibrium by the gradual formation of another no longer cleavable lactam group.

The chlorophyll component *a* is allomerized in various ways, for example, as follows:



Since the ring groupings that are newly formed by the first attack of the alkalies upon the chlorophyll are more stable than the original ones, a variation in the course of the relactamization results in permanent differences in the products of further decomposition. Therefore, from the two fundamental forms of each chlorophyll component, chlorophyllin and iso-chlorophyllin, there are derived corresponding series of more simply composed phyllins and porphyrins. In the case of alkali decomposition, therefore, there are produced, in all, four series which all lead finally to a single end-product.

When heated with concentrated methyl alcoholic potash in a closed vessel the chlorophyllin molecule, without being split up, is simplified by a further transformation of its acid-containing groups; heating the phytochlorins and phytorhodins with magnesia and alkali leads to the same result. The beautifully crystallizing phyllins, which resemble one another are formed in this way; they are dicarboxylic acids that are either isomeric or differ only by two hydrogen atoms, and monocarboxylic acids, all with complexly bound magnesium. They are derived from a common parent substance:



$$C_{31}H_{34}N_4Mg,$$

which shall be called etiophyllin, by one or two of its hydrogen atoms being substituted by a carboxyl. Their magnesium-free derivatives, the porphyrins, which are characterized by their basic properties and can be differentiated by means of these are carboxylic acids of etioporphyrin:


$C_{31}H_{36}N_4$.

From iso-chlorophyllin *a* are formed:

the di-carboxylic acid, cyanophyllin, blue in solution;
the di-carboxylic acid, erythrophyllin, red in solution;
the mono-carboxylic acid, phyllophyllin,[44] bluish red in solution;

From chlorophyllin *a* arise:

the di-carboxylic acid, glaucophyllin, blue in solution;
the di-carboxylic acid, rhodophyllin, blue-red in solution;
the mono-carboxylic acid, pyrrophyllin, bluish red in solution.

The carboxyls, α and β, are probably present in glauco- and rhodophyllin, while γ and β are present in cyanophyllin and in erythrophyllin; phyllophyllin, accordingly, is the monocarboxylic acid with the γ carboxyl while pyrrophyllin contains the α carboxyl.

Corresponding decomposition of the chlorophyll component *b* was made difficult by the tendency of its derivatives to form amorphous products of high molecular weight; the decomposition also takes a somewhat more complicated course, since two oxygen-containing groups must be transformed in this case into simple pyrrol nuclei.

The di-basic phyllins differ from those formed from the *a* component but the same mono-carboxylic acids are finally obtained; pyrrophyllin from chlorophyllin *b* and phyllophyllin from iso-chlorophyllin *b*.

The connection between the phyllins and the porphyrins and their relations to the first chlorophyll derivatives are disclosed by the table on the following pages.

Decarboxylation of all chlorophyll derivatives on heating with alkalies in an autoclave leads to the monocarboxylic acids only since decomposition ensues at higher temperatures. The removal of the last carboxyl was finally accomplished by using the classical soda-lime method, in the case of the phyllins as well as with the porphyrins. It is most successful if a very small amount of a porphyrin—in the case of a phyllin it runs still more smoothly—is heated for a short time with soda-lime. The same etioporphyrin is obtained in this manner from phylloporphyrin as from pyrroporphyrin and the same etiophyl-

[44] See page 334 regarding the derivation of the words used.

Phäophytin a
$[C_{32}H_{33}ON_4]$ (CO_2CH_3) $(CO_2C_{20}H_{39})$

mit Säure ← / → mit $MgCH_3I$

Chlorophyll a
$[C_{31}H_{29}N_3Mg]$ $(NH \cdot CO)$ (CO_2CH_3) $(CO_2C_{20}H_{39})$
γ γ β α

beim Stehen in Alkohol →

Allom. Chlorophyll a
$[C_{32}H_{30}ON_4Mg]$ (CO_2CH_3) $(CO_2C_{20}H_{39})$

mit Alkali, verdünnt

mit $CH_3OH-HCl$ ↓

mit Enzym ↓

Methylphäophorbid a
$[C_{32}H_{33}ON_4]$ (CO_2CH_3) (CO_2CH_3)

← mit Säure

Methylchlorophyllid a
$[C_{32}H_{30}ON_4Mg]$ (CO_2CH_3) (CO_2CH_3)

mit Alkali, heiß

mit Alkali, kalt

mit Alkali, heiß und kalt

mit Alkali, verdünnt

mit kons. HCl ↓

mit Enzym ↓

mit Alkali

Phäophorbid a
$[C_{32}H_{33}ON_4]$ (CO_2CH_3) (CO_2H)

← mit Säure

Chlorophyllid a
$[C_{32}H_{30}ON_4Mg]$ (CO_2CH_3) (CO_2H)

mit Alkali, heiß | mit Alkali, kalt

mit Alkali ↓

Phytochlorin e
$[C_{32}H_{33}ON_4]$ (CO_2H) (CO_2H)

mit Säure ← / → mit MgO

Iso-Chlorophyllin a
$[C_{31}H_{29}N_3Mg]$ $(NH \cdot CO)$ (CO_2H) (CO_2H)
γ α β γ

Chlorophyllin a
$[C_{31}H_{29}N_3Mg]$ $(NH \cdot CO)$ (CO_2H) (CO_2H)
δ γ β α

mit Säure →

Phytochlorin f und g
$[C_{32}H_{33}ON_4]$ (CO_2H) (CO_2H)

mit Alkali auf 140° erhitzt ↓

mit Alkali auf 140° erhitzt ↓

mit Alkali auf 150° erhitzt

Cyanoporphyrin
$[C_{31}H_{34}N_4]$ $(CO_2H)_2$

← mit Säure

Cyanophyllin
$[C_{31}H_{32}N_4Mg]$ $(CO_2H)_2$

Glaukophyllin
$[C_{31}H_{32}N_4Mg]$ $(CO_2H)_2$

mit Säure →

Glaukoporphyrin
$[C_{31}H_{34}N_4]$ $(CO_2H)_2$

mit Alkali ↓

mit Alkali auf 170° erhitzt ↓

mit Alkali auf 165° erhitzt ↓

mit Alkali ↓

Erythroporphyrin
$[C_{31}H_{34}N_4]$ $(CO_2H)_2$

← mit Säure

Erythrophyllin
$[C_{31}H_{32}N_4Mg]$ $(CO_2H)_2$

Rhodophyllin
$[C_{31}H_{32}N_4Mg]$ $(CO_2H)_2$

mit Säure →

Rhodoporphyrin
$[C_{31}H_{34}N_4]$ $(CO_2H)_2$

mit Alkali ↓

mit Alkali auf 190° erhitzt ↓

mit Natronkalk

mit Alkali auf 200° erhitzt ↓

mit Alkali ↓

Phylloporphyrin
$[C_{31}H_{34}N_4]$ (CO_2H)

← mit Säure / →

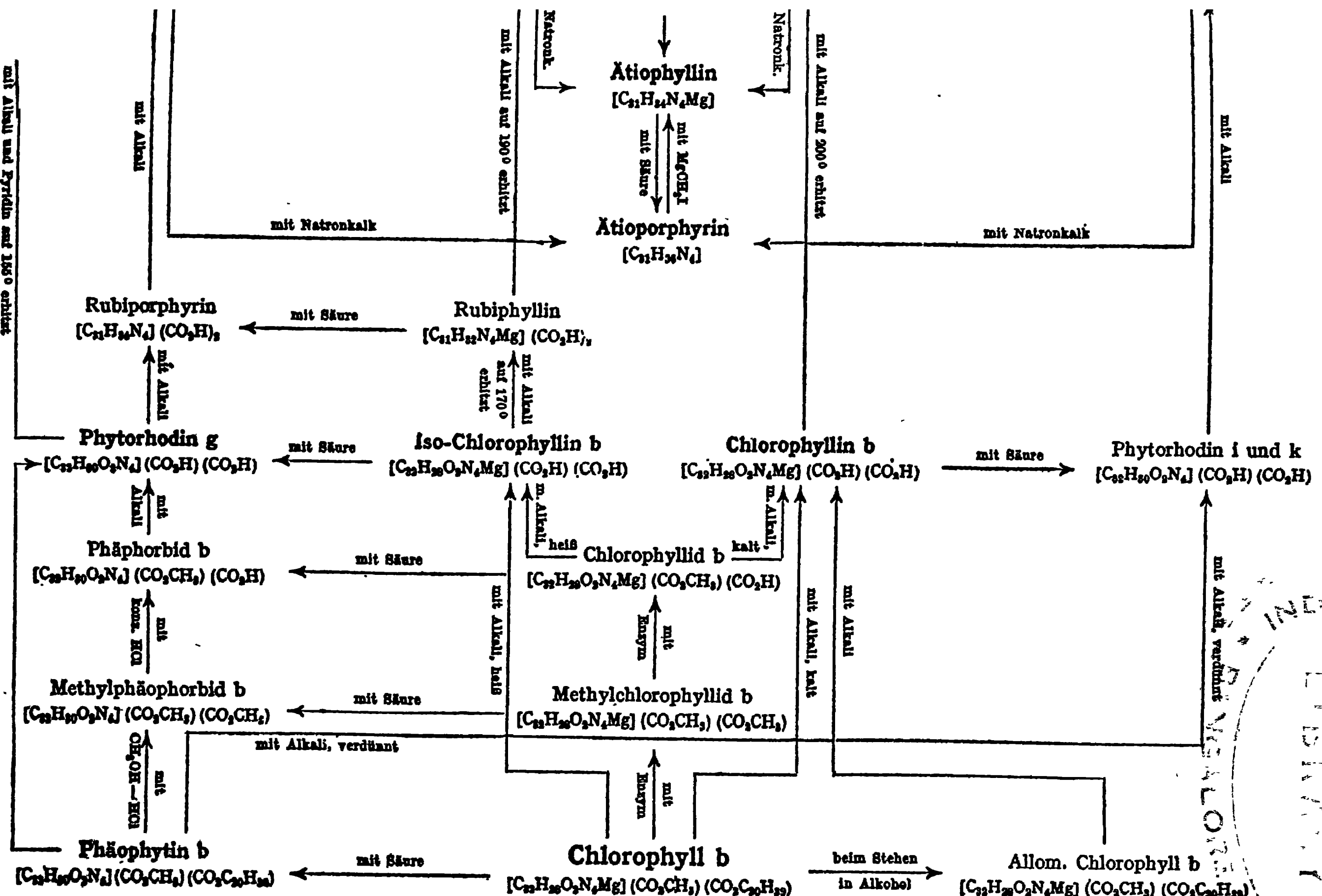
Phyllophyllin
[illegible]

Pyrrophyllin
[illegible]

mit Säure →

Pyrroporphyrin

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lin is obtained from their magnesium compounds. That the difference between the isomeric monocarboxylic acids is conditioned only by the position of the carboxyl is confirmed by this.

The parent substances, crystalline compounds, fit into the group picture that has been sketched with the numerous phyllins and porphyrins that were previously investigated. They open up new possibilities for future investigations on systematic decomposition. They are also important because identical transformation products whose molecules are still closely related to the dyestuffs themselves are now obtained for the first time from leaf pigment and from blood pigment by means of them.

Up to this time only such decomposition products as were produced by the oxidation and reduction of hemin and chlorophyll, the simpler pyrrol derivatives, have been identical, while the porphyrins from both pigments exhibited many similarities and many differences.

In an investigation by Willstätter and M. Fischer, which we will report here briefly and in more detail elsewhere, the decomposition of the well-known hematoporphyrin, which contains 6 oxygen atoms, was carried through to its oxygen-free compound.

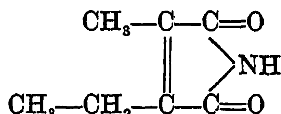
The porphyrins are formed from hemin by entirely different reactions than from chlorophyll but not in so simple a manner as by removal of the metal since hematoporphyrin is a dihydroxy acid. It was successfully reduced to a new porphyrin, which is called hemoporphyrin, by heating with methyl alcoholic potash and pyridine. This differs from hematoporphyrin in its composition by the absence of two hydroxyl groups; it contains possibly two atoms less of hydrogen than does mesoporphyrin. Hemoporphyrin, from its analysis and properties, is closely related to rhodoporphyrin and erythroporphyrin and, like these, is decarboxylated on heating with soda-lime. Since the magnesium compounds lose their carbon dioxide more easily, magnesium is first introduced into the hemoporphyrin and the metal is then finally split off from the carboxyl-free compound. The reaction product is identical with the etioporphyrin from chlorophyll as regards composition and properties such as its spectrum and its basicity which was tested quantitatively by means of its distribution between ether and hydrochloric acid.

From the composition, $C_{31}H_{36}N_4$, of etioporphyrin it follows that hemoporphyrin corresponds to the formula, $C_{33}H_{36}O_4N_4$, and consequently hemin does not correspond to the generally adopted formula, $C_{34}H_{32}O_4N_4FeCl$, but to the formula, $C_{33}H_{32}O_4N_4FeCl$. This is, in

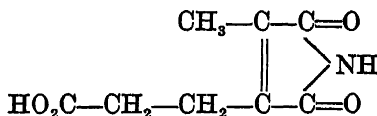
fact, in agreement with the important analytical results for hemin to which attention was called some time ago.[45] Independently of the decomposition of etioporphyrin, it has been found from the analyses of a great number of porphyrins derived from hemin that the formulae of hemin and hematoporphyrin are very probably to be altered in this way.

In order to find an expression for the structure of the parent substance, etioporphyrin, the results of the oxidation and the reduction of the various porphyrins, which are described in more detail in two later chapters, must be considered.

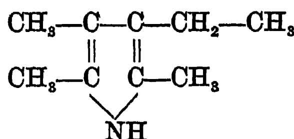
Phylloporphyrin produces on oxidation more than one molecule of methylethylmaleic imid:



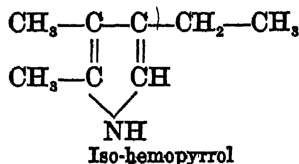
and a molecule of hematic acid:



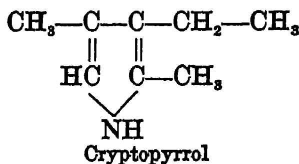
On reduction the porphyrins give mixtures of pyrrol homologs, among which are contained especially:



Phyllopyrrol



Iso-hemopyrrol



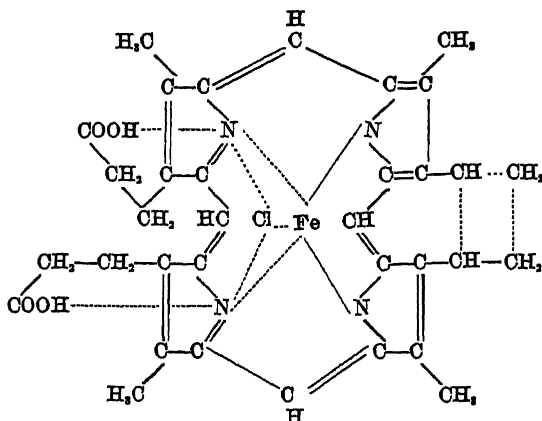
Cryptopyrrol

Etioporphyrin is accordingly composed of four pyrrol nuclei. The number of its hydrogen atoms is strikingly low, which implies that the pyrrols must, therefore, be so linked and substituted that in comparison

[45] Ann. d. Chem. 358: 212. 1907.

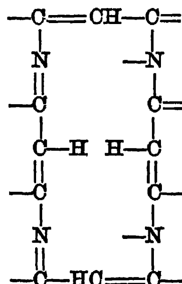
with simple linkages eight hydrogen atoms are saved, either by double linkage or by further closing of rings. Several ideas in regard to the manner in which one can consider the pyrrol nuclei bound are met with in the literature on hemin.

W. Küster[46] has illuminated the subject of blood pigment by the following constitutional formula for hemin, which is in accordance with the course of oxidation and reduction:



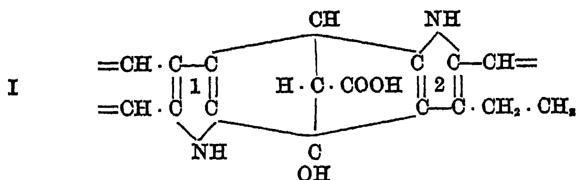
Hemin $C_{34}H_{32}O_4N_4FeCl$.

It, however, has the objection that, of the two imino groups that are linked with iron only one is represented as an acid imid of a pyrrol; the other, however, as a basic imino group of a dihydropyrrol. The improbability of this formula lies chiefly in the assumption of a ring of sixteen atoms consisting of four nitrogen atoms and twelve carbon atoms:

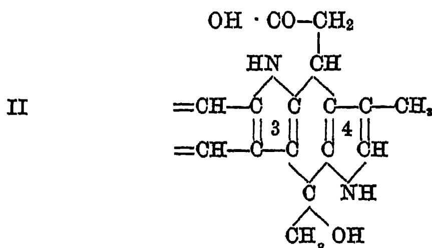


[46] Zeitschr. f. physiol. Chem. 82: 463. 1912.

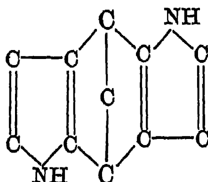
The first changes of such a complicated pigment are so difficult to unravel that the views of different investigators vary widely. O. Piloty[47] traced hemin back to two condensed systems which are linked together and which consist of pyrrols with six and five carbon atom rings:

I 

and

II 

This conception is possible only on the assumption that reduction easily decomposes the cyclopentane and cyclohexane rings. This is hardly probable.[48] The contradiction between the results of oxidation and the essential idea of these assumed constitutions is still more important. Hematic acid can not be obtained from hemin and its derivatives according to the formulae of Piloty. The tertiary carbon atoms of both formulae, for example

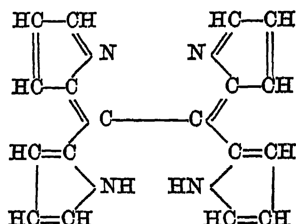


make the formation of the propionic acid residue of hematic acid impossible; the same objection may be raised against the formula of bilirubinic acid as given by O. Piloty and S. J. Thannhauser.[49]

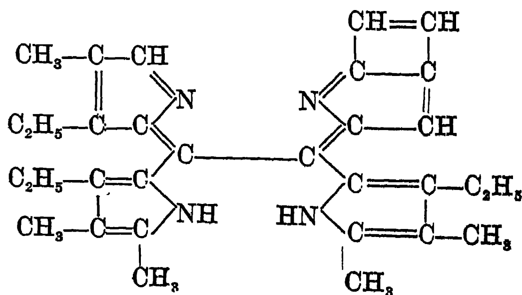
[47] O. Piloty and E. Dormann. Ann. d. Chem. 388: 313. 1912.
[48] Cf. H. Fischer and E. Bartholomäus. Zeitschr. f. physiol. Chem. 83: 50, 1912.
[49] Ann. d. Chem. 390: 191. 1912.

The peculiar ring formations which the formulae that are adduced present are a result of the difficulty of expressing the union of the pyrrols by a molecule so poor in hydrogen. It just does not appear possible, if the ethyl groups necessary for an explanation of the oxidation products are provided, to formulate the etioporphyrin without the assumption of a carbon ring.

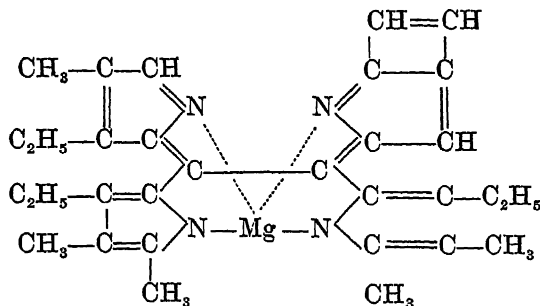
The following formula, in which two of the nuclei are salt-forming and two are complex-forming, appears to us to be a probable representation of the simple union of the four pyrrol nuclei to form a pigment:



If, as a result of a consideration of the oxidation products, three methyl and three ethyl groups are substituted in this parent substance, and at least another methyl group, as a result of a consideration of the reduction products, there finally remains only so much hydrogen for the last three carbon atoms of the etioporphyrin that either two double bonds or two carbon rings or one of each must be assumed. If, in conformity with the course of reduction, the assumption of a cyclopentane or cyclohexane ring is avoided, we arrive with some probability at the following formulae for etioporphyrin and etiophyllin:



Etioporphyrin $C_{31}H_{36}N_4$.



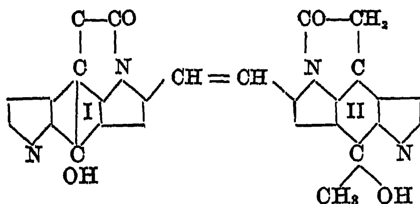
Etiophyllin $C_{31}H_{34}N_4Mg$.

Several details of this formula, such as the position of two methyl groups, are arbitrary. The cyclobutane ring could also be attached to the pyrrol in the β-β-position.

The carboxyls of phyllo- and pyrroporphyrin replace ethyl groups; the formula is unsymmetrical enough to satisfy the observed isomers of the acid porphyrins.

If an attempt is now made to explain the relation between etioporphyrin and hemin, the questions presented by the first changes of hemin are still confronted.

Willstätter and Fritzsche[50] called attention four years ago to the fact that, according to the investigations of M. Nencki and J. Zaleski[51] on ester formation, two free carboxyls exist in hemin. W. Küster[52] supported this view with fundamental evidence, while O. Piloty clings to the assumption that hemin contains the two carboxyls in the form of lactam groups and the two remaining oxygen atoms as hydroxyls:



However, the analyses of the dialkylester by Nencke and Zaleski, whose results correspond to formulae like $C_{32}H_{30}(COOCH_3)_2N_4FeCl$,

50 Ann. d. Chem. 371: 49. 1909.

51 Zeitschr. f. physiol. Chem. 30: 384. 1900.

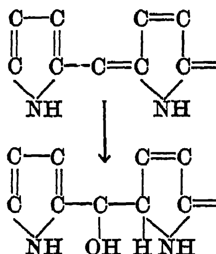
52 Zeitschr. f. physiol. Chem. 66: 165. 1910. Zeitschr. f. physiol. Chem. 86: 185. 1913. Ber. d. deutsch. chem. Ges. 45: 1935 and 2503. 1912.

and also the oxidation of dimethylhemin to hematic acid ester according to the method of Küster are opposed to this lactam formula.

In the elimination of the iron from the hemin there occurs simultaneously, according to Piloty, a dissolution of the assumed lactam groups; hematoporphyrin accordingly would be the iron-free compound, which corresponds exactly to hemin. On the other hand, W. Küster considers two vinyl groups in hemin as the points for attack by reagents, for example, by hydrobromic-glacial acetic acid, in the elimination of the iron and H. Fischer[53] considers it probable that double bonds between methine groups and pyrrol nuclei take up the hydrogen halide, and thus, indirectly, water.

Willstätter and M. Fischer conducted experiments on the course of hematoporphyrin formation and discovered a series of intermediate products which show that two molecules of hydrogen bromide are first taken up and that the linkage of the iron is considerably loosened by this.

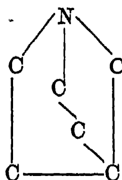
The explanation of H. Fischer would cause one to expect that the color of the compounds would be affected or toned down by the additions:



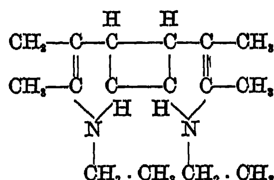
which is not the case. According to the view of Küster additions would occur at unimportant groups which do not impair the complex linking.

The assumption that those groups that are characterized by a pronounced tendency to take up additions are in combination with nitrogen atoms (somewhat according to the following scheme for which many alkaloids offer analogies) appears to us well founded for the explanation of the reactions involved in the elimination of the iron:

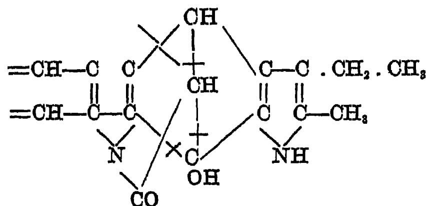
[53] H. Fischer, E. Bartholomäus and H. Röse, Zeitschr. f. physiol. Chem. 84: 262, 1913.



This formula for a portion of the hemin molecule agrees very well with a noteworthy, but yet unconfirmed, observation of O. Piloty and J. Stock[54] who found among the hemopyrrols a polymerized pyrrol in the form of its picrate, probably ethylated at the nitrogen, that is, a bis-α-β-dimethyl-N-ethyl-pyrrol of the formula:



It is improbable that the ethyl group arises from the carboxyl that is present as a lactam and a carbon atom of the five carbon atom ring, as Piloty and Stock assume:



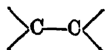
Even though etioporphyrin, of the formula, $C_{31}H_{36}N_4$, appears strikingly poor in hydrogen, hemin, $C_{33}H_{32}O_4N_4FeCl$, is derived from a parent substance,


$$C_{31}H_{34}N_4,$$

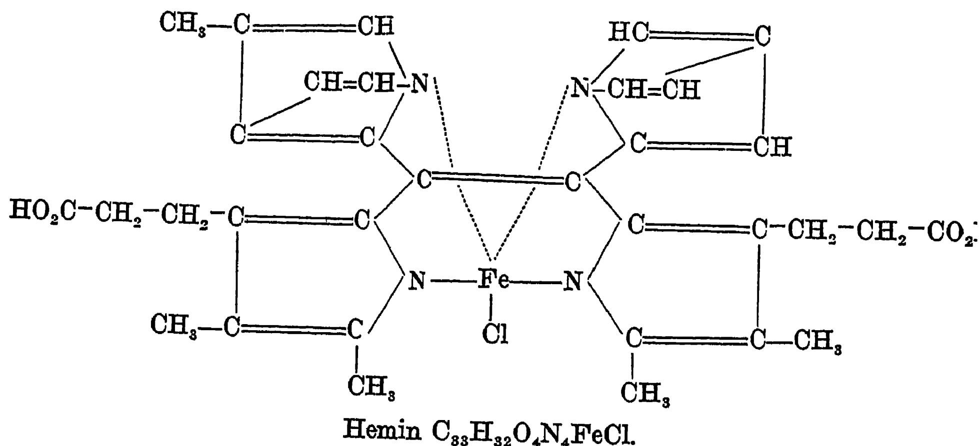
still poorer by two hydrogen atoms, which makes necessary the assumption of double bonds and carbon rings.

[54] Ann. d. Chem. 392: 215, 1912; see also O. Piloty and K. Wilke, Ber. d. deutsch. chem. Ges. 46: 1597, 1913.

Upon the basis of the assumed connection of four pyrrols by the group

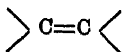


and of the union of two vinyls to nitrogen atoms an attempt is made by us to develop a satisfactory constitutional formula[55] of hemin that will accord with its behavior on oxidation and reduction and on porphyrin formation. This formula still lacks proof as to several details but it is hoped that it will serve to stimulate further investigations.

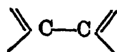


Hemin $C_{33}H_{32}O_4N_4FeCl$.

In the formation of hematoporphyrin the connecting links joining the two pyrrol nitrogens will become detached so that the middle group



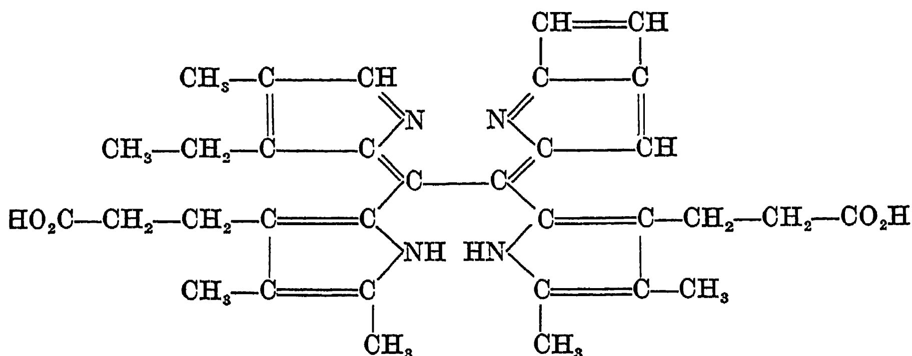
can then change into



In transformations of hematoporphyrin, condensation of a vinyl radicle should take place, according to our assumption, with a carbon atom of the pyrrol nucleus. This gives expression to the structural

[55] Ann. d. Chem. 400: 182, 1913.

image of etioporphyrin. Hemoporphyrin is therefore illustrated by the following formula:



Hemoporphyrin $C_{33}H_{36}O_4N_4$.

and mesoporphyrin, which possibly contains two more hydrogen atoms, perhaps by the corresponding formula with the CH═CH group saturated.

The formulae disclose that the molecule of hemin changes on decomposition in an unsymmetrical manner, as is indicated by several reactions. Although on oxidation no methylethylmaleic-imid appears; mesoporphyrin furnishes[56] this imid, though not more than one molecule.

A supposition in our considerations is the assumption of the simple molecular magnitude of hemin and hematoporphyrin, the formulae of which are expressed with 33 carbon atoms. Although molecular weight determinations[57] of numerous chlorophyll derivatives and J. Zaleski's[58] determinations for mesoporphyrin are in harmony with this assumption, O. Piloty and E. Dormann[59] have drawn the conclusion from experiments on the elevation of the boiling point of pyridine solutions of hematoporphyrin, "that the molecular weight of hematoporphyrin must be assumed to be double that which has been

[56] H. Fischer and F. Meyer-Betz. Zeitschr. f. physiol. Chem. 82: 96, 1912; also W. Küster and P. Deihle, Ber. d. deutsch. chem. Ges. 45: 1935, 1945, 1912, and Zeitschr. f. physiol. Chem. 82: 463, 1912.

[57] Ann. d. Chem. 382: 155, 1911.

[58] Zeitschr. f. physiol. Chem. 37: 73, 1902.

[59] Ann. d. Chem. 388: 319, 1912.

heretofore assumed''; this deduction was supported by O. Piloty and H. Fink[60] and extended to hemin and hemoglobin. These conclusions, however, are not to be considered correct,[61] for Willstätter and M. Fischer have confirmed the simple molecular weight with the beautifully crystallized tetra-methyl compound of hematoporphyrin (dimethyl-ether-dimethyl-ester) in veratrol solution.

Although chlorophyll and hemin have been traced back to the same etioporphyrin, which may be designated a parent substance, we do not conclude from investigations on their decomposition that a close constitutional relationship exists between chlorophyll and hemin. In the one there is magnesium, in the other, iron; in one, ester formation with phytol, in the other, a combination with globin. In addition to such differences which correspond to dissimilar functions there are further important differences in the nucleus of the pigment itself which disappear only upon far-reaching decomposition.

Two transformations which essentially alter the structure of the molecule take place in the change from hemin to etioporphyrin, namely, the transition from hemin to hematoporphyrin and from this to hemoporphyrin, which is isomeric with the porphyrins of chlorophyll.

Also between chlorophyll and etiophyllin and even between chlorophyll and the first dibasic porphyrins, such as cyanoporphyrin or erythroporphyrin, there are two steps which radically alter the form of the molecule and which are not paralleled by the reactions of hemin.

One step is the change in lactam rings of the chlorophyll components by which one ring system is broken and a new one is formed. The second transformation of the molecule, which is less obvious, occurs as a result of the action of alkali at a higher temperature and leads from the chlorophyllins to the more simply constituted dibasic phyllins or from phytochlorin and phytorhodin to the corresponding porphyrins.

The formation of cyanoporphyrin from phytochlorin appears to proceed simply by the splitting off of a carboxyl according to the equation:



$$C_{34}H_{36}O_6N_4 = C_{33}H_{36}O_4N_4 + CO_2.$$

[60] Ber. d. deutsch. chem. Ges. 45: 2495, 1912, and 46: 2020, 1913.

[61] Either the preparation which was used was not good or hematoporphyrin cannot withstand boiling with pyridine, as it is also damaged on drying at 100°.

But since the porphyrins are all similar, although very different from the chlorins and rhodins, the transformation is probably a more radical one. If the lactam in phytochlorin were formed from a carboxyl substituted in the pyrrol and the pyrrol nitrogen, the splitting off of carbon dioxide would hardly influence the properties much, possibly as little as the passage from the dibasic to the monobasic porphyrins. It is more probable, therefore, that the lactam group belongs to the class of pyridones, its carboxyl being, therefore, itself a ring constituent and that the fourth pyrrol nucleus of cyanoporphyrin is formed only upon the elimination of a carbon atom.

Future investigations on the constitution of chlorophyll, will, therefore, still find important problems. The relations between the two chlorophyll components and the transformations which lead from chlorophyll to the chlorophyllins and from the chlorophyllins to the dibasic phyllins are yet to be explained and important details of the structure of etioporphyrin remain to be investigated.

II. DESCRIPTION OF THE LEAF PIGMENT BY MEANS OF SIMPLE EXPERIMENTS.

The extraction of chlorophyll, its separation from the accompanying yellow pigments, its isolation and preparation in the form of beautifully crystallized derivatives, namely, methyl and ethyl chlorophyllides, and its transformations are dealt with in the following chapters. Some simple experiments which may be adapted to work on a small scale, for instance, for plant physiology lectures and for botanical practice, are based upon the methods described in these chapters. These experiments will serve to illustrate the separations of the leaf pigment and its properties.

The following chief facts form a basis for these experiments:

The chloroplasts contain four pigments; namely, two closely related chlorophyll pigments and two yellow pigments, mixed in a colloidal state with colorless substances:

The chlorophyll component *a*, of the composition, $C_{55}H_{72}O_5N_4Mg$, blue black and in solution greenish blue.

The chlorophyll component *b*, of the composition, $C_{55}H_{70}O_6N_4Mg$, green black and in solution pure green.

Carotin, of the formula, $C_{40}H_{56}$, orange red crystals.

Xanthophyll, of the formula, $C_{40}H_{56}O_2$, yellow crystals.

These pigments have been found to be identical in all the plants of the different classes which have been investigated and in the comparative investigation it has been found that chlorophyll is characterized by its constant composition.

It contains 2.7 per cent magnesium and gives an ash of pure magnesia (without iron or phosphorus).

Upon saponification it yields the nitrogen-free alcohol, phytol, ($C_{20}H_{40}O$), as a third of the molecule.

The nitrogen-containing complex, which contains four pyrrol nuclei, is identified by the cleavage product, phytochlorin *e*, from the component *a*, and phytorhodin *g* from the component *b*. The former is olive green in ether; the latter is red.

Fresh leaves contain about 2 parts of chlorophyll *a* in a thousand, 3/4 of a part of *b*, 1/3 of a part of xanthophyll and 1/6 of a part of carotin.

Behavior toward solvents.

The methods recommended in botanical books for the preparation of chlorophyll solutions are not suitable.

Experiment 1. 10 g. fresh leaves (stinging nettle) are comminuted in a mortar with a little sea-sand and transferred to a funnel provided with a sieve-plate and filter-paper. Then, 20 cc. of acetone are poured upon the mass and filtered off, using a filter-pump. The pouring on and sucking off of the solvent are repeated. The filtrate (about 40 cc.) contains 0.02 g. of chlorophyll.

Experiment 2. On immersing a leaf in boiling water for a short time it is colored a more vivid green and a microscopical section shows that the chloroplasts have disintegrated. The oxidizing enzymes have been destroyed as a result of this treatment. Although fresh elder or horse chestnut leaf turns olive brown on grinding and leaves a brown mass on extraction, a scalded leaf remains green when ground and the leaf substance is white after the extraction.

Experiment 3. Powdered dry leaves do not give up the pigment to benzol and only very slowly to absolute alcohol, ether and acetone, although they are extracted well by 80 to 90 per cent alcohol or acetone. Petroleum ether does not extract chlorophyll at all.

Experiment 4. 2 g. of dried stinging nettle leaves are pulverized and sucked fast on a suction filter. Then, 10–20 cc. of 85 per cent (by volume) acetone (or 90 per cent alcohol) are poured on slowly in small portions with occasional slight suction by means of the pump and in a moment almost all the pigment is obtained in the beautiful green, intensely red fluorescent extract.

The Effects of Chlorophyllase.

Experiment 5. Fresh, disintegrated leaves (Heracleum, Galeopsis), rich in chlorophyllase, are steeped in 70 per cent (by volume) acetone (1 g. per 3 cc.). Considerable chlorophyll comes out of the leaf substance; it is split up by the action of the enzyme into phytol and the acid chlorophyllide. After the lapse of a quarter of an hour it can be seen that this has occurred, if the pigment is diluted with water and extracted with ether, by shaking the ethereal solution with 0.05 per cent sodium hydroxide. The more complete the enzyme action the greater is the quantity of pigment taken up by the hydroxide.

Experiment 6. Steeped leaves of the same chlorophyllase-rich plants produce with aqueous acetone under the same conditions a solution of unaltered chlorophyll, which does not react with the dilute sodium hydroxide. By this means it is shown that hydrolysis (and alcoholysis) is an enzyme reaction.

Experiment 7. A number of microscopical sections of a Heracleum leaf are moistened on a slide with a drop of 90 per cent alcohol and covered with a cover-glass. The slide is permitted to remain beside a small vessel of alcohol underneath a small bell jar until it has dried (one-half to a whole day). Then, as stated by Borodin, there may be seen in the cells and beside the leaf tissue beautiful triangular and hexagonal forms of crystallized chlorophyll (ethyl chlorophyllide) (Fig. 7, on page 159).

Experiment 8. Four cc. of 75 per cent methyl alcohol are poured upon a gram of a fresh Heracleum leaf in a test-tube. The leaf first becomes deeper green and then changes to yellow during the course of one to three hours. The chlorophyll which leaves the leaf has lost its phytol; the methyl chlorophyllide that is produced forms black spots in the leaf tissue; these appear as glittering clusters of crystals under the microscope. (See Fig. 6, on page 158.)

Experiment 9. Two grams of powdered dry leaves (Heracleum) are allowed to stand with 6 cc. of 90 per cent alcohol in a test tube for a half to a whole day. The extract is then filtered on a small suction filter, and the meal subsequently washed with some acetone. The filtrate is mixed with an equal volume of ether and with water, and the ethereal solution of ethyl chlorophyllide is retained in a test-tube size separatory funnel (Fig. 1) and washed with water. The ethereal solution is concentrated to 0.5 to 1 cc. in the separatory funnel on a water-bath and 3 cc. of petroleum ether are added to the warm solution. The chlorophyll precipitates, on standing, in crystalline aggregates. It is freed from yellow pigments by shaking with a little ether and then recrystallized from more ether.



Fig. 1. Test-tube dropping funnel.

Characteristics of Chlorophyll.

Experiment 10. An acetone extract of fresh leaves obtained by the procedure of experiment 1 is diluted with 5 times its volume of 85 per cent acetone in order to observe its

absorption spectrum. For this, a 1 cm. layer in a glass vessel with parallel walls is used. Sunlight or a Welsbach lamp is used as the source of light. We recommend for the observation a pocket grating spectroscope made by Fuess, of Steglitz, with a scale of wave-lengths.

The spectrum shows a principal absorption band in the red at the Fraunhofer line C; three absorption bands then follow, with decreasing intensity, toward the violet end, and, finally, a second absorption maximum which is marked by complete absorption of the blue to violet region (Fig. 1 of Plate I).

On adding a drop of hydrochloric acid to a test portion of the diluted acetone extract the color of the chlorophyll changes immediately to olive green. The magnesium complex is destroyed and the spectrum of the pheophytin formed shows an intensive absorption band in the green before the line E (Fig. 2 of Plate I).

Experiment 11. An ethereal solution of leaf pigment is prepared from the acetone extract of dried leaves (Experiment 4) by pouring it into 30 cc. of ether contained in a separatory funnel and then adding 50 cc. of water. By this means the ethereal layer separates. It is washed four more times, each time with 50 cc. of water which is allowed to run down the walls of the vessel without shaking. The ethereal solution (15 cc.) is easily emulsified to a certain extent and can be cleared by shaking with anhydrous sodium sulphate and filtering.

Chlorophyll, as an ester, exhibits no acid properties; the ethereal solution does not react with aqueous alkali on gentle shaking.

The characteristic brown phase, which Molisch discovered, appears upon placing a layer of 30 per cent methyl alcoholic potash under the ethereal layer. At the surface of contact there appears immediately a beautiful brown ring which permeates the solution on shaking. In about 10 minutes it changes again through an intermediate olive to pure green. The chlorophyll has been saponified to form the potassium salt of the acid, chlorophyllin; on dilution with water the green color will consequently no longer go into the ethereal layer.

Experiment 12. The decomposition of chlorophyll to phytochlorin and phytorhodin and the separation of these components can be carried out with 5 cc. of the ethereal solution obtained above (Experiment 11).

The ether is removed by evaporation in a test-tube and 3 cc. of boiling methyl alcoholic potash is poured upon the residue, which is boiled gently a half minute longer over a Bunsen burner. The potassium salt

solution, which shows a beautiful red fluorescence, is diluted with double its volume of water and neutralized with concentrated hydrochloric acid till the reaction is just distinctly acid. The cleavage products formed yield an ethereal solution with an olive brown color on shaking with ether in a separatory funnel of test-tube size.

For the separation of the cleavage products, the ethereal solution is shaken twice, each time with about 10 cc. of 4 per cent hydrochloric acid; the greenish-blue acid layer gives, upon neutralization with ammonia and extraction with ether, an olive green solution of phytochlorin *e* whose source is the chlorophyll component *a*.

The portion remaining after the extraction with 4 per cent acid is extracted once with 10 cc. of 12 per cent hydrochloric acid; the green acid solution, on dilution with water, gives up phytorhodin *g*, the derivative from the component *b*, to the ether, in which it dissolves with a red color.

Experiment 13. Two cc. of an ethereal solution of crude chlorophyll are shaken with a little 20 per cent hydrochloric acid and then with some water; the decanted ethereal solution of the magnesium-free chlorophyll derivative is evaporated on a water-bath and the residue is taken up with 5 cc. of alcohol. The olive colored fluid assumes, on warming with a small crystal of copper acetate, a beautiful chlorophyll color in consequence of the formation of a copper compound, similar to the leaf pigment but more stable.

Separation of the Pigments.

Experiment 14. Five cc. of the ethereal solution of the leaf pigment (Experiment 11) are shaken strongly with 2 cc. of concentrated methyl alcoholic potash. After the green color has returned the solution is gradually diluted with 10 cc. of water and a little more ether is added. After thorough shaking in a test-tube two layers form, the aqueous alkaline layer contains the chlorophyll and the ethereal layer the carotinoids.

Experiment 15. The ethereal solution of carotinoids obtained in the preceding experiment serves for the separation of the yellow pigments. It is separated in a separatory funnel, washed with water and evaporated to 1 cc. It is then diluted with 10 cc. of petroleum ether and extracted about three times with 90 per cent methyl alcohol, using 10 cc. each time, till the methyl alcoholic layer no longer becomes col-

ored. The xanthophyll is in the methyl alcohol and the carotin is in the petroleum ether. The absorption spectra of the yellow pigments (5 mg. in 1 liter of ether, 1 cm. thick), pictured in Figure 4 of Plate 1, are observed with a pocket grating spectroscope.

Experiment 16. All the pigment from a fourth of the acetone extract of 2 g. of dried leaves (Experiment 4) is transferred to petroleum ether by extraction with 10 cc. of petroleum ether and 20 cc. of water in a test-tube separatory funnel. If the petroleum ether solution, which has been washed with a little water, is thoroughly shaken with 10 cc. of 92 per cent methyl alcohol, the chlorophyll *b* and xanthophyll are extracted by the alcohol while chlorophyll *a* and carotin remain in the petroleum ether.

The yellow accompanying pigments diminish the color difference between the chlorophyll components; their dissimilarity becomes clearly visible in the color change caused by placing a layer of concentrated methyl alcoholic potash under the solutions. In order to carry out the test (as in Experiment 11) chlorophyll *b* is again dissolved in ether by dilution and extraction. The chlorophyll component *a* gives a yellow and *b* a brownish red phase.

The characteristic differences (see Chapter VI) between the spectra of the perfectly pure chlorophyll components (solution of 0.04 g. in 1 liter ether, layer 2 cm. thick) are shown in Figure 3 of Plate I.

III. THE EXTRACTION OF THE PIGMENTS.

1. Plant Material.

Fresh and dried leaves are used as initial material. Earlier authors almost always worked with fresh plants, viz., they generally extracted grass with boiling alcohol.[1] It is often recommended, for example, by R. Sachsse[2] and A. Tschirch,[3] that the fresh leaves be first thoroughly boiled in water, then pressed and extracted with warm alcohol. F. Hoppe-Seyler[4] washed the grass with ether in order to remove the wax before extraction with boiling alcohol.

A statement on the use of dried plants is given by A. Hansen,[5] who first boiled fresh grass for a quarter to a half hour in water, then dried it and, without grinding, extracted with boiling alcohol.

For most preparative purposes we use dried leaves in a powdered form and we always carry out the extraction at room temperature.

This procedure has the following advantages: the volume and the weight of the leaves are much smaller so that vessels one tenth as large may be used.

Less of the solvents is required, for they are not diluted by the water content of the leaves (70–80 per cent of the fresh weight).

Preparation does not depend on the time of the year or on the habitat of the plant.

Comminution is easier.

The elaboration of leaf meal makes it possible to obtain chlorophyll and its derivatives in technical quantities and facilitates their production in the laboratory in such quantities as are required for chemical investigation.

[1] E. Schunck. Proc. Roy Soc. 39: 348, 1885 and 44: 448, 1888.

[2] Phytochem. Investigations. I. Chem. Untersuchungen über Chlorophyll. Leipzig, 1880.

[3] Untersuchungen über das Chlorophyll. Berlin, 1884. Also Ber. d. d. bot. Ges. 14: 76, 1896.

[4] Zeitschr. f. physiol. Chem. 3: 339, 1879.

[5] Die Farbstoffe des Chlorophylls. Darmstadt, 1889. Page 46.

Since the yields of the preparations no longer represent a small unknown fraction but most of the chlorophyll present in the plant, information concerning yields is given for all the methods of preparation. This has hitherto been entirely lacking in the literature.

Two disadvantages are to be considered in drying the plant material, loss of chlorophyll and decomposition of the pigments.

The decrease of pigment content is very noticeable with commercial leaves, the pigment content of which fluctuates according to the care bestowed in drying and the weather prevailing during the collecting season.

While fresh stinging nettle leaves (with stems) contain 8–10 g. of chlorophyll per kilogram (calculated on the dry basis), the chlorophyll of commercial nettle meal customarily amounts to only 5–6.5 grams. The leaf material thereof may also be diluted by worthless portions of the plant.

If we do the drying ourselves (this is best done over a steam boiler or in an oven) no chlorophyll is lost. Willstätter and Utzinger found in alcoholic extracts of dried stinging nettle and Galeopsis leaves 95–96 per cent of the chlorophyll of the fresh leaves.

The dry weight of the plants, for the most part 25 per cent of the fresh leaves (for stinging nettles), is influenced by the season of the year and by the growing conditions.

Plant	Time of year	Growing conditions	Dry weight per cent
Stinging nettle	March 22	....	17.5
Stinging nettle	July 20	....	34.0
Elder	July 12, 5 p.m.	sun leaves	27.8
Elder	July 11, 5 p.m.	shade leaves	16.3
Horsechestnut	July 17, 7 a.m.	sun leaves	37.5
Horsechestnut	July 17, 7 a.m.	shade leaves	25.0
Pine	July 21	....	50.0

Many dry leaves, for example, grass, spoil easily on storage, others (elder, needles of conifers) spoil easily even on drying. It was found possible to dry even such leaves with the preservation of their chlorophyll by placing them in quantities of 50–100 g. in a vacuum desiccator over sulphuric acid.

Brown algae, for example, Fucus, were especially difficult to dry without injury. Even after steeping for a short time, centrifuging and spreading out in a warm air current they can not be ground to a beautiful green meal nor can the pigments be extracted with a good yield.

The second disadvantage, which may occur in the use of dried leaves and is often feared by botanists—the decomposition of the pigments on drying—does not easily occur and can be avoided by suitable choice of plants and proper drying.

We have determined the characteristics of chlorophyll and found them the same in comparative investigations with fresh and dried plants.

The working up of fresh leaves is especially important analytically, for example, for the quick isolation of pure chlorophyll from small quantities of leaves and, furthermore, for the quantitative determination of the two green and the two yellow pigments.

The use of fresh material is important also in the case where the action of chlorophyllase upon chlorophyll is utilized, hence, in obtaining crystalline chlorophyll. In fact, preparation of the free chlorophyllides has been successful up to now with fresh leaves only, and for a long time this was true also of the preparation of the methyl chlorophyllides. Then, imitating the working conditions which occur with fresh leaves, the method was applied to dry leaves.

Finally, it was found very advantageous in the case of the brown algae, which are especially difficult to preserve, to use them in the fresh state for the preparation of fucoxanthin and chlorophyll.

Fresh leaves are in many respects much more difficult to work up than dried ones; for example, to grind and to protect against alterations of the chlorophyll. In such cases, a preliminary treatment according to the method of Willstätter and Isler[6] with aqueous methyl or ethyl alcohol, of such concentration that no chlorophyll is extracted, is of value.

The leaves are dehydrated, hardened and easily pulverized by this treatment. For best results they are treated with as much absolute methyl alcohol as will be diluted by the water content of the leaf to about 66 per cent (by volume); then more 66 per cent (by volume) methyl alcohol is added till the leaves, which are weighted down mod-

[6] Ann. d. Chem. 380: 171, 1911. See also, Chapter VII, Section 3.

erately by stones, remain wholly immersed in the fluid. It is often advantageous to use mixtures of aqueous methyl alcohol and ether, whereby much wax is extracted from certain plants.

In this manner beautiful chlorophyll solutions can be very successfully produced from fresh pine-needles which can scarcely be worked up otherwise; for example, by treating 800 g. of pine needles with 1500 cc. of methyl alcohol, 900 cc. of water and 600 cc. of ether.

After this treatment the chlorophyll is much more easily extracted than ordinarily and with the proper length of treatment the yield is usually satisfactory.

Plant	Duration of Treatment	Chlorophyll in grams from 1 kg. fresh leaves
Stinging nettle	1–18 hours	2.1 to 1.6
Pine needles	2 hours	0.9
Equisetum	18 hours	1.4
Fern	2 hours	1.5
Moss	16 hours	1.4

Treatment in aqueous methyl alcohol is also applicable to the meal from dry leaves in order to make its chlorophyll more easily extractable.

Classes of Plants. In the choice of plants we differentiate between those rich in chlorophyllase, which are suitable only for the preparation of crystalline chlorophyll, as: Heracleum spondylium, Galeopsis tetrahit, Stachys silvatica, and those poor in chlorophyllase which are best for most preparative purposes, as for the preparation of chlorophyll, pheophytin, phytol, chlorophyllin salts and further derivatives.

We generally use stinging nettles, as they are cheap, rich in chlorophyll and poor in enzymes, can be well dried, and remain green on storage. For the preparation of pure chlorophyll we find it advantageous to do the drying ourselves because a more favorable degree of purity as well as a better yield can be obtained from material rich in chlorophyll. Commercial nettle leaves, which are purchasable as average fine meal at the price of 65–80 marks for 100 kg., are good enough for the preparation of pheophytin. The meal usually contains only 7 per cent of moisture.

The use of stinging nettles can be traced back to G. G. Stokes,[7] who wrote about it in his celebrated treatise of the year 1852, "On the Change of Refrangibility of Light," though in no later publication:

"A good number of the following observations on the internal dispersion of leaf-green were made with a solution obtained from the leaves of the common nettle, by first boiling them in water and then treating them with cold alcohol, the leaves having previously been partially dried by pressing them between sheets of blotting-paper. Nettle was chosen partly because it stands boiling without losing its green color, and partly for other reasons."

Stinging nettles have, however, the disadvantage that the chlorophyll in their extract alters very easily in such a way that weakly basic cleavage products appear upon systematic decomposition instead of the normal ones. This rarely happens in the case of the extract from other plants. This change can be avoided by quick extraction and immediate elaboration of the extract.

Example. Five hundred g. of stinging nettle meal was extracted a short time by the suction filter method with 95 per cent alcohol. Half of the filtrate was placed for 2–5 days in the dark or in the light. The preparation of pheophytin and its saponification resulted, in addition to the phytochlorin *e*, in much phytochlorin *f*, while the phytorhodin *g* was deficient.

The second half of the stinging nettle extract was mixed with the extracted meal and allowed to stand the same length of time. Systematic decomposition gave a normal mixture of chlorin *e* and rhodin *g* without more weakly basic admixtures.

2. Methods of Extraction.

(*a*) *State and Behavior of Chlorophyll in Leaves.*

Our chief departure from the usual method of operation of earlier authors consists in extraction at room temperature, for which the meal of dry leaves is suitable.

The solvents applicable to the extraction are alcohol, methyl alcohol, ether and acetone.

Their use is by no means conditioned only by the solubility of the chlorophyll. Chlorophyll in the pure state is, as we now know, easily soluble in benzene and in anhydrous acetone, but is not extractable by

[7] Edinburgh Transactions 12: 486, 1852.

them. It is easily soluble in petroleum ether while mixed with accompanying materials, yet it can not be extracted at all by its use.

Absolute alcohol extracts the dry leaf meal only slowly; ether and chloroform, as well as acetone, very difficultly; benzene, petroleum ether and carbon disulfid, not at all; on the contrary, methyl alcohol extracts it immediately.

Willstätter and Mieg[8] first made extractions with solvents that do not take up chlorophyll (petroleum ether, benzene and carbon disulfid) for the isolation of carotin and the method afterwards served us for some time for the preparation of purer chlorophyll solutions because of the removal of a greater quantity of colorless and yellow accompanying substances. The advantage of this preliminary treatment to the purity of the chlorophyll extract should by no means be judged solely by the amount of material dissolved by it; the effect of the preliminary extraction is seen rather in the petroleum ether chlorophyll solutions, which are not obtained until the pigments of the extract are separated by means of petroleum ether. The solvents suitable for preliminary treatment remove about 17 g. of extractive material from a kilogram of stinging nettle meal.

Example. One kg. of stinging nettles dried at 40° C. was placed upon a suction filter and quickly extracted with several solvents, each of which was fully displaced by the succeeding one:

1. Two liters anhydrous acetone dissolved 0.9 to 1.0 g. of chlorophyll and gave 15.4 g. of residue.
2. Six liters benzene gave 3.5 g. residue.
3. One liter ether dissolved 0.05 g. chlorophyll and contained 0.8 g. residue.
4. Petroleum ether: almost no action.
5. One liter anhydrous acetone took up 0.15 g. of chlorophyll, with much yellow, and gave 1.65 g. of residue.

4.7 g. of pure chlorophyll was then obtained by working up with aqueous acetone.

We sought formerly to explain the peculiar behavior toward solvents of chlorophyll in leaves by the assumption that the chlorophyll probably occurred in the leaf substance in the form of adsorption compounds with colloids; A. Arnaud[9] similarly assumed that it is held back

[8] Ann. d. Chem. 355: 12, 1907.

[9] Chemisches Zentralblatt; an abstract. 100: 751, 1885.

in the leaf tissues by capillary attraction and M. Tswett[10] assumed that the pigment is bound to the framework of the chloroplasts by molecular adsorptive forces.

Our observations do not confirm W. Palladin's[11] assumption, based upon the behavior of the leaves toward petroleum ether, that the chlorophyll is contained in the leaves in a chemically combined condition; namely, bound with phosphatides (lipoids). We can decolorize solutions of chlorophyll with animal charcoal and also by means of extracted plant meal and the pigment then occurs in the form of an adsorption product which, for example, is not affected by petroleum ether. It is even difficult to extract the chlorophyll from the animal charcoal again but it can be done with pyridine.

The use of solvents for the extraction of the leaf green may therefore be explained by the dissociative power with which they act upon the adsorption products.

Recent experiments cause us to prefer another concept of the state of the chlorophyll in leaf tissue.

All the absorption bands in the spectrum of a living leaf are, according to M. Tswett[12] and previous authors, displaced with respect to the spectrum of a chlorophyll extract toward the more weakly refracted end. D. Iwanowski[13] compared the spectra of leaves and of colloidal chlorophyll solutions. He finds them similar to each other but not identical and assumes that the chlorophyll in the leaf is not dissolved as a colloid but is present as a fine suspension. A. Herlitzka[14] points out, on the other hand, the coincidence of the spectra of living leaves with those of colloidal solutions of chlorophyll and their common difference from real chlorophyll solutions. He concludes that even if there is not an identity there is still a similarity in the condition of the chlorophyll in leaf tissues and in colloidal solution.

Our own experiments approximately confirm the conclusions of A. Herlitzka; we have measured the spectra of leaves of different plants and find them identical as regards the position of the absorption bands with the spectrum of a colloidal solution of pure chlorophyll *a* (Chapter VI, section 4), although they differ somewhat in the relative intensities of the bands.

10 Arbeiten der Naturf. Ges. Kasan. 35: 86, 1901.
11 Biochem. Zeitschr. 26: 357, 1910 and Ber. d. d. bot. ges. 28: 120, 1910.
12 Die Chromophyll in der Pflanzen- und Tierwelt. Pg. 173, Warsaw, 1910.
13 Ber. d. d. bot. Ges. 25: 416, 1908 and Biochem. Zeitschr. 48: 328, 1913.
14 Biochem. Zeitschr. 38: 321, 1912.

These spectroscopical measurements and especially the observations on the behavior of leaves toward solvents make it probable that chlorophyll is present in leaves in a colloidal state of distribution or in a very similar condition.

This state of the chlorophyll is influenced in a characteristic manner by steeping the leaves; the chlorophyll is then more easily extracted.

While normal leaf tissue contains its chloroplasts nicely arranged along the cell walls (more closely packed in the palisade cells, more scattered in the spongy parenchyma) and, indeed, in sharply defined, mostly ellipsoidal forms, leaves, after the short action of boiling water, show the chloroplasts either strongly deformed or burst so that their somewhat granular masses have coalesced and diffusely filled the cells. The diffusion takes place almost immediately. The leaves are colored deep green a few seconds after steeping in boiling water, a phenomenon that is most beautiful with the brown algae.

Spectroscopically, this color change of the leaves is seen as a displacement of the absorption bands toward the violet end of the spectrum, their position approximates somewhat that observed in the spectrum of a chlorophyll extract and differs but little from the position of the bands shown by a solution of chlorophyll mixture in phytol.

The chlorophyll has gone over from a colloidal condition to a form of true solution, that is, it has dissolved in the waxy accompanying substances which have liquefied as a consequence of the rise in temperature. It has become easily soluble and even benzene easily extracts the pigment from the meal of steeped leaves.

On steeping leaves their chlorophyll goes into solution in a strongly refractive medium.

A chlorophyll solution can also be formed within the leaf tissue with the same solvents as are used for its extraction; for example, a leaf of stinging nettle is placed in acetone till it appears uniformly deep green and no chlorophyll has yet passed from the cells (see Chapter VII, section 2); spectroscopical measurements then give values which coincide as to the position and intensity of the bands with those of the spectrum of an extract.

We have assembled some of our spectroscopical observations in the following table; for the measurements given we passed light by means of a Nernst lamp and a condenser through the stinging nettle leaves

placed directly in front of the slit (0.1 mm. wide) of a Zeiss grating spectroscope.

Depth of layer in mm.	Living leaf	Steeped leaf	Solution of chlorophyll in phytol	Leaf treated with acetone
Band I	693—663	686—657	685—654	
" II	. . 643	. . 645	. . . 641	680—640
" III	625 | 611	623 | 608	625 . . 603	625 . . . 601
" IV	592 . 569	590 . 569	590 . . 570	588 . . 564
" V	551 . 535	550 | 535	548 . 532	548 . 526
" VI	520 . . . 505—	519 . . . 505—	512 | 486	514 . . . 502—
End absorption			. . . 480—	

These comparative measurements explain fully the state and the optical relations of the chlorophyll in the living leaf, as well as in the leaf which has been treated with hot water or with solvents and has become a brighter green.

When leaves are kept in an ice chest no change of the chloroplasts takes place that is observable in microscopical sections but when these leaves are placed in aqueous methyl alcohol they show a different behavior than that exhibited by freshly plucked ones. They lose their color more rapidly; for example, stinging nettle leaves in 5 instead of 21 hours.

In this treatment of the leaves[15] the chlorophyll passes out of the chloroplasts and precipitates elsewhere in the leaf; probably the solvent first forms with the accompanying material of the chlorophyll a mixture which acts upon the pigment as a good solvent and then precipitates it again upon increasing dilution by the solvent.

The meal from stinging nettle leaves that have been dried after storage in the ice chest does not give up its chlorophyll to petroleum ether, but gives it up easily to benzene so that it can be precipitated by means of petroleum ether from the solution that is formed; absolute alcohol, water-free acetone, and ether also extract the pigment from it very easily, and petroleum ether with an unusually small addition of alcohol also does.

The cause of this phenomenon has not yet been explained; perhaps it depends upon an alteration of the colloids in the chloroplasts as a

[15] Compare Chapter VII, Section 2.

result of changes of concentration due to the loss of water from the cell constituents.

The difficult solubility of the chlorophyll that is contained in the uninjured leaf substance, and the fact that it becomes easily soluble under the circumstances stated, appeared to indicate that the chlorophyll perhaps occurs in the leaf in the state of a loose chemical compound, but it has not been possible for us to find any support for such a conjecture.

Chlorophyll is obtained without any difference in its solubility relations or optical properties no matter whether it is quickly extracted and isolated from fresh leaves or on the other hand prepared from leaves which have been steeped and stored in the cold.

A good explanation for the remarkable relative solubilities of the pigment on treatment of the leaf meal with water-free solvents and, on the other hand, with solvents in the presence of water is offered by the assumption of a colloidal state of the chlorophyll in the leaf tissue.

Dry meal of stinging nettle leaves does not color acetone in half an hour, but it does so immediately and intensively when a little water is present. Absolute alcohol behaves similarly, but the difference here is less marked. Methyl alcohol acts just oppositely; when it contains water it dissolves the chlorophyll poorly while absolute methyl alcohol dissolves it immediately and well. Ether and benzene are not colored by leaf meal; they are still free from chlorophyll after 5 minutes, but if the meal is moistened with a few drops of water the ether is at once colored a strong green, and benzene is similarly but a little more slowly.

Colloidal aqueous solutions of chlorophyll, whether prepared from extracts[16] or from pure chlorophyll, do not give up any chlorophyll to ether or benzene when shaken with them but they give it all up immediately on the addition of a little salt; for example, calcium chloride.

We believe that the behavior of chlorophyll in leaf meal is similar to this: The water added to the organic solvents dissolves mineral salts, as for example, potassium nitrate, from the leaf substance. The salt solution that is formed changes the colloidal state of the chlorophyll in the chloroplasts and makes it easily soluble.

[16] Paper II.

This circumstance is of great value in the extraction of pigments from dried leaves by means of aqueous solvents.

(b) *Our Older Methods*

The extracts are defined by their chlorophyll content and by its degree of purity.[17] We term the ratio of the chlorophyll to the entire quantity of material, generally dissolved substances, expressed in per cent, the degree of purity. By means of this value the influence of methods of preparation and of purifying operations upon the separation of chlorophyll from the colorless and yellow accompanying substances is tested.

The degree of purity of solutions is obtained from the colorimetrically ascertained chlorophyll content and from the dry residue, which is determined by evaporation on a water-bath and heating in a vacuum to constant weight; with quantities of 1–2 grams constant weight is generally reached in 30–45 minutes.

The yellow pigments of the leaf, as well as fats, waxes, phytosterin, carbohydrates, salts (*e.g.*, KNO_3) and other colorless substances, are contained in the crude chlorophyll solutions in addition to the green pigment. The dry residue from an extract of one kilogram of stinging nettle leaves amounts to 30–40 grams or more; solutions of such small concentration produce a resinous residue, dirty in color, which puffs up in a vacuum while solutions with a high percentage of chlorophyll give a brittle blue-black residue which, after drying, dissolves easily with a beautiful green color.

Bottle Extracts.

One kg. of leaf meal is extracted in a stoppered bottle with 2 l. of alcohol, which requires, at most, a few hours of mechanical shaking. The extract is filtered by means of a pump, the powder is strongly pressed on the suction filter and washed till the volume of the filtrate equals 2 l. (the volume started with).

In preparative work on a larger scale, stronger extracts, so-called double extracts, are made because the alcoholic or ethereal extracts serve again for the extraction of leaf powder.

Double extracts.[18] Each 50 kg. of moderately fine meal from dry

[17] Ann. d. Chem. 380: 184, 1911.

[18] Ann. d. Chem. 350: 65, 1906.

stinging nettle plants or grass was shaken to a uniform paste with 75 liters of alcohol (96 per cent) in a dozen 12-liter powder bottles. The extraction was complete after 24 hours standing, particularly when the contents of the bottles had been very thoroughly mixed by frequent rolling of the bottles upon a thick felt mat and by repeated vigorous shaking. The solution was then thoroughly removed in three portions by using a suction pump and a large, stoneware suction filter which was covered with a plate. Since the pressed powder retains, on an average, 0.8 l. of the extract per kilogram, washing with 40 l. of alcohol was necessary for the preparation of the extract; the wash alcohol displaced the extract from the powder without diluting it. Thus, 75 l. of simple extract were obtained. A second washing was then made with about 20 l. of alcohol in order to fully leach the powder; this wash alcohol yielded a very dilute solution of chlorophyll, which was simply used for mixing with fresh powder.

A second charge of 50 kg. of nettle leaves was extracted with this first extract in about 48 hours. Filtering of the extract with subsequent washing took place in the same manner, except that it was advantageous to use double the quantity of alcohol, about 40 l., for the last washing of the double extract. There was obtained, accordingly, from 100 kg. of leaves and 155 l. of alcohol, 75 l. of double extract; a further 60 l. of wash alcohol which contained chlorophyll were obtained and used for the preparation of the next extract.

Percolates[19]

Glass percolators[20] ranging from 0.5 to 1 l. up to 12 to 25 l. in capacity are used. Figure 2 shows a percolation apparatus of 4 units, each of which holds 25 l., in operation.

Larger percolators of stoneware are more durable and cheaper than the glass ones but they are difficult to handle on account of their great weight.

Before the plant powder is placed in the percolator it is moistened with alcohol (0.3 l. per kg.), mixed well and allowed to stand 3–4 hours in covered wooden vessels. After this time has elapsed, the

[19] R. Willstätter. Chlorophyll in E. Abderhalden's Handbuch der biochem. Arbeitsmethoden 2: 674, 1910; also Ann. d. chem. 378: 5, 1910.

[20] The percolators may be purchased from the glass factories of Poncet, Inc., Berlin.

powder is run through a horse-hair sieve (1.5 mm. mesh) and then placed in the percolators. The bottom of the vessel is first covered with a thin layer of wadding, which acts as a filter. The material must be put in rather loosely but as uniformly as possible and then tamped lightly. If it is too tightly pressed it clogs easily; on the other hand, if the charge is not uniform and is too loosely placed, the alcohol finds channels through which it flows off without extracting. The

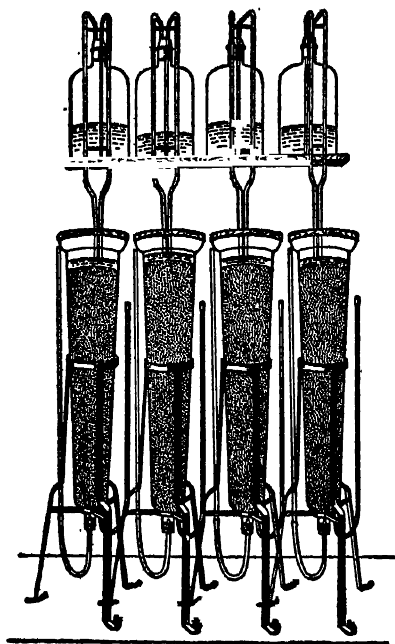


Fig. 2.

lower limit of the solvent, as it sinks in, should form an almost horizontal circle.

About 2 l. of alcohol per kg. of nettle powder are required for percolation and subsequent washing. With percolators of 3 kg. capacity (10–12 l. water content) a bottle of 6 l. content is simply turned upside down and placed upon the upper rim of the percolator; it empties at the rate at which the alcohol sinks downward. For large percolators (holding about 10 kg. stinging nettle leaves), bottles containing 17 l. of alcohol each, are set up above. They have ground-

in caps through which a glass tube passes almost to the bottom of the bottle while a second tube opens in the cap; both lead into the percolator to just above the charge. In this manner the flow of the alcohol is automatically regulated. The percolator is closed by a ground-glass plate, provided with a slot, to avoid the absorption of moisture.

It usually requires 12–15 hours for the alcohol to penetrate through the entire charge of the large percolators. The maceration is usually not carried any further than this but the percolate is allowed to drop into a flask or else sucked off. The run off is at first very highly concentrated; it gradually becomes more dilute and it is advantageous to change the receiver when 0.6 to 0.7 l. of extract per kg. of material has run off from a large percolator; that is, after approximately 24 hours. A further runoff which amounts to about 0.30 to 0.45 l. per kg. of plant meal is, in the course of 8–10 hours, drawn by suction into the second receiver. This second run off serves for addition to the next charge.

Example. A charge of 36 kg. of powder is macerated with 10 l. of alcohol and percolated in the 4 units of the apparatus with 16 l. of second runnings and 47 l. of fresh spirit; 23 l. of percolate flows freely into the first receiver; 16 l. of second runnings are sucked off into the second receiver. The meal retains 34 l. of alcohol which can be distilled off.

The extraction of chlorophyll by means of percolators continues of merit even when contrasted with the different improvements in the method which are described in section *d* of this chapter.

The First Suction Filter Method.

The slow extraction in bottles and in percolators presents two disadvantages:

1. Alcoholysis under the influence of chlorophyllase causes a loss of phytol which is not insignificant even with stinging nettles although they belong to enzyme-poor plants.

With slow extraction the phytol number of nettle leaves was 28.2 (an average of determinations made on 12 preparations) while with quick extraction it was 32.2.

2. The chlorophyll changes on long standing of the solution (especially easily in the case of stinging nettle extracts) in such a way that cleavage of the pheophytin gives rise to the weakly basic phytochlorin *f* instead of the normal phytochlorin *e*.

Hence, it is advantageous to make use of a shorter method of extraction; namely, the extraction of the powder directly upon a suction filter. One kg. of Galeopsis meal is moistened in a porcelain dish for 5 minutes with 0.5 l. alcohol (96 per cent), worked till homogeneous and then placed in a thick layer upon a suction filter. 0.5 l. of alcohol is now poured on it and suction by means of the vacuum pump is begun at once. Finally, alcohol is alternately poured on and sucked off till about another liter is used. Twenty minutes from the time that the leaves have been moistened 1 l. of alcoholic extract, which contains 5 g. of chlorophyll, is obtained.

In preparative work this suction filter method has the disadvantage of smaller yields; these increase with the duration of the extraction; for instance, the following yields are obtained by extracting 1 kg. of stinging nettle leaves upon a suction filter:

Time of extraction	Cc. of extract	Chlorophyll, in grams
15 min.	700	2.9
19 "	800	3.3
25 "	850	3.7
120 "	800	4.4
3 days	6000	7.1 (quantitative extraction)

The best results for a 2 kg. charge on a suction filter are obtained, therefore, when the duration of the extraction is 2–3 hours. The quantity of solvent need not be greater than in the preparation of double extracts; 1.5 l. per kilogram of meal is sufficient.

When working with the percolator the material must be dried between the preliminary extraction and the extraction; this tedious operation, which has an unfavorable action on the chlorophyll, cannot be avoided because clogging would otherwise take place during the extraction of the leaf substance, which has become compact during the preliminary handling. On the other hand, when the suction filter is used, the preliminary extraction and the extraction are simply combined. This method of extraction, therefore, presents, apart from its simplicity, an especial advantage in the combination of the preliminary extraction with different solvents and the extraction with alcohol.

When working with large quantities the leaf powder is simply placed upon the suction filter without being first moistened with alco-

hol. The meal is uniformly distributed upon the suction filter during continuous suction with the vacuum machine. With some practice and careful work, one may fill even a large stoneware suction filter with 20 kg. of powder without any irregularities occurring in the extraction.

The following observations show that the method and the duration of the extraction exert but little influence upon the degree of purity of the chlorophyll, much less than differences in the nettles harvested.

Extraction of 1 kg. of commercial stinging nettle meal	Time of extraction	Cc. of extract	Residue in grams	Degree of purity	Chlorophyll in grams
1. Percolates					
a. long	48 hrs.	1000	33.6	16	5.5
b. short, 1st run off	1 "	120	4.9	12	0.6
c. short, 2nd runnings	2 "	220	12.3	12	1.5
2. Flask extracts					
a. double extraction	48 hrs.	1060	34.8	16	5.5
b. simple extraction	48 "	1600	46.6	13	6.2
c. quick extraction	10 min.	1300	22.0	13	2.8
d. quick extraction	30 "	1600	21.6	16	3.5
3. Suction filter extracts					
a. quick	15 min.	500	12.5	17	2.1
b. moderately quick	2 hrs.	800	31.4	14	4.4

A preliminary treatment with benzene is most favorable for the degree of purity of the chlorophyll; one with petroleum ether is less favorable, but since the petroleum ether solution must be free from benzene for the isolation of chlorophyll the benzene of the preliminary extraction is displaced from the leaf meal by means of petroleum ether. Each kilogram of leaf meal required 3 l. of benzol and 1 to 1.5 l. of petroleum ether and the extraction of a charge of 2 kg. required 2–3 hours.

The following table shows the yield and the degree of purity of the chlorophyll that has been carried over in the petroleum ether. Experiments 1–4 refer to the crude petroleum ether solution, No. 5 refers to the same solution after it has been washed once with one-half its volume of 90 per cent methyl alcohol. The figures of number 5 are the averages from four experiments.

Number	Preliminary extraction	Extract	Extract			Petroleum ether chlorophyll solution		
			Chlorophyll in g.	Residue in g.	Degree of purity	Chlorophyll in g.	Residue in g.	Degree of purity
1	None	Alcohol	4.9	37.3	13	3.5	11.2	31
2	None	Methyl alcohol	3.7	38.8	10	2.4	8.8	27
3	Petroleum ether	Alcohol	5.3	35.0	15	3.6	9.8	37
4	Petroleum ether	Methyl alcohol	3.7	41.2	9	2.3	8.2	28
5	Benzene	Alcohol	6.1	43.7	14	3.9	8.0	49

(*c*) *Fundamental Principles of the New Method of Extraction*[21]

The use of solvents with a considerable water content is an unexpected improvement of great importance in comparison to the extraction methods above described.

The state of the colloidally dissolved chlorophyll in the chloroplasts is changed by the salt solution (for example KNO_3) that is formed with the use of aqueous solvents. It becomes easily soluble.

Besides, the quantity of the accompanying substances going into solution is increased. The real extraction medium for the green pigment of the leaf is no longer the solvent itself but its mixture with the accompanying materials and so excellent a solvent is it that the pigments are quickly and easily extracted by it almost quantitatively. It appears as if the whole chloroplast substance were carried away immediately by a solvent with suitable water content.

The accompanying substances of the pigments are to be distinguished; some are easily soluble in petroleum ether and difficultly soluble in aqueous solvents and others are of reverse solubility. Water-free solvents extract many accompanying substances of chlorophyll which resemble it in their solubility relations and consequently follow it persistently. On the other hand, the aqueous extracts are poor in those accompanying substances that pass over with the chlorophyll into the petroleum ether on separation from the extracting solvent, and also in those that precipitate when the chlorophyll is precipitated with water or when the pheophytin is separated.

[21] Unpublished.

Quantitative Determinations. In order to compare quantitatively the action of the most important solvents, both when water-free and with varying water content, and to find the best conditions for extraction the method of procedure was strictly observed as to the quantities of plant meal and solvent used and the duration of the extraction.

It would be possible to extract all the pigment with each extraction medium, even when this is water-free, by prolonging sufficiently the duration of the experiment and making unlimited use of the solvent. For purposes of comparison the amount of solvent is regularly so chosen as to be sufficient for a complete extraction under the most favorable conditions and the duration adopted is the time necessary for this. Consequently, with the use of some water-free solvents the filtration must be delayed in order to obtain a uniform experimental procedure because these of themselves run through too quickly. In order to obtain equal volumes of extracts it must still be remembered that the solvents are unequally retained by the plant meal; those containing water are held back most.

The differences in the chlorophyll content would be increased if only the first half of the extracts were compared; in the case of aqueous acetone the first half already contains the greater portion of the pigment, while extraction proceeds uniformly from beginning to end when the water-free solvents are used. But with a view to a method for the preparation of chlorophyll comparison of the total extraction is more important.

In the following comparison of the extractions with alcohol, acetone, methyl alcohol and ether, a quantity of stinging nettle leaves collected by us during the early part of May was used. This (15.5 kg.), after drying and grinding, was spread out for several days at 30–40° to dry fully. In this way 900 g. of moisture were lost; the material for the experiments then contained only 1.3 per cent of moisture and consequently did not color benzol.

0.5 kg. of this stinging nettle meal was spread upon a filter cloth in a suction filter (24 cm. inside diameter) and was tamped fast under suction of two water-filter pumps. 500 cc. of solvent were now poured on, without suction, and allowed to sink into the meal for five minutes. 250 cc. more were then added, and about the same suction was applied with a filter-pump in all the experiments after 50 cc. of the particular water-free solvent used had been placed in the filter-flask in order to prevent considerable evaporation of the solvent during the period of

extraction. The suction used is so slight that the solvent in the flask does not come to a brisk boil. Another 250 cc. were added after five minutes and suction applied for ten more minutes. Two more successive quarter liters were allowed to flow through, ten minutes being required for each, and, finally, strong suction was applied with both filter-pumps. This much time is required on account of the swelling of the leaf substance when treated with water-containing solvents.

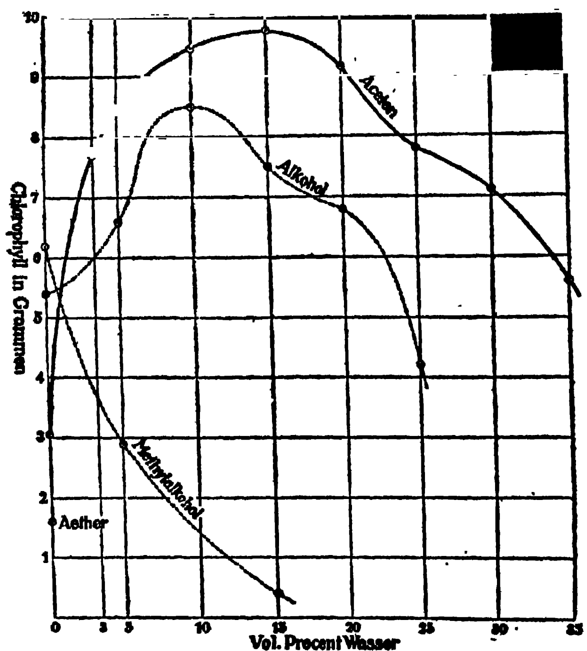


Fig. 3.

We always seek to obtain 0.8 to 0.9 l. of extract from 1.5 l. of solvent. With water-free solvents that much is easily obtained and tho experiment is then discontinued; with solvents containing much water a somewhat smaller volume causes no error, because here the last part of the extract is very poor in chlorophyll.

If at times some crude chlorophyll separated in the suction flask it was dissolved in acetone and united with the extracts. In most cases the volumes of the extracts were brought to 1 l. and the chlorophyll estimated colorimetrically by saponification with alkali and compari-

son with crystallized chlorophyll in accordance with the method given in Chapter IV, section 1.

The results of the comparison, which are but slightly influenced by the conditions of the experiment and the plant material, are contained in the following table and the graphic presentation of Figure 3 gives a clear picture of them. The number of determinations are, of course, not sufficient to fix the curves exactly as to details but their courses have been confirmed by a repeated series of experiments.

Table.

The stinging nettle leaves used contained 10 g. of chlorophyll in 1 kg. of dry meal.

Volume per cent	Grams of chlorophyll in the extract			
	Acetone	Ethyl alcohol	Methyl alcohol	Ether
100	3.05	5.4	6.2	1.6
97	7.70	——	——	——
95	——	6.6	2.9	——
90	9.45	8.5	——	——
85	9.75	7.5	0.4	——
80	9.15	6.8	——	——
75	7.80	4.2	——	——
70	7.10	——	——	——
65	5.60	——	——	——

Although the water-free solvents arrange themselves in the following series: ether, acetone, ethyl alcohol and methyl alcohol, an addition of even 1 per cent of water causes acetone, methyl alcohol and ethyl alcohol to have the same extractive effect (5.25 g. of chlorophyll are dissolved). A greater water content reverses the series and ethyl alcohol is surpassed by acetone.

Our methods for the isolation of chlorophyll and its accompanying pigments and for the preparation of pheophytin are based upon these results.

Extraction with acetone requires a water content of 10–20 per cent. The optimum is at about 15 per cent water by volume. Eighty per cent acetone is used for the extraction, however, and just as favorable a chlorophyll yield is then obtained by using a little more solvent.

With acetone that contains a higher percentage of water the quantity of accompanying materials that are soluble in petroleum ether is lessened and the isolation of the chlorophyll is made easier.

The optimum for alcohol was found to be a water content of 10 per cent by volume. This applies also to commercial stinging nettle meal, which is important for the preparation of pheophytin, and it is extracted with 90 per cent alcohol for this purpose.

For purposes of comparison, 1 kg. lots of commercial stinging nettle meal which contained only 5.1 g. of chlorophyll per kilogram were extracted, without further drying, upon a suction funnel with 1.5 l. of alcohol the water content of which varied for each lot; with the finer, heavier material which, however, is poorer in pigment, this volume is sufficient. The chlorophyll content of the extract (0.9 l.) was with

95 per cent alcohol......	4.28 grams
90 " " "	5.04 "
85 " " "	4.67 "

(d) Method of Extraction with Aqueous Solvents[22]

The Procedure for Dried Leaves. Extraction according to the new method is made by means of a suction filter according to the above described suction filter method.

The chief condition for carrying out the method is the use of thin layers of leaf meal so that the extract does not become too concentrated and sirupy. Otherwise, chlorophyll and other substances separate in the deeper layers of the powder; this hinders filtration and can stop it entirely. A deeper layer has the additional disadvantage that the concentrated extract formed removes more and more of the fat and wax, which decreases the purity of the chlorophyll solution. We therefore never use (regardless of whether the leaf meal is rich or poor in chlorophyll, whether fine or coarse and, therefore, less dense), layers that are thicker than 4–5 cm. when sucked tight, regardless of whether the suction filter is very large in diameter or not.

Since the stoneware suction filters that are technically used are unsuitable for such procedure, we had the Ton- und Steinzeugwerken, A.–G., Charlottenburg, manufacture suction filters of 50 centimeter inside diameter, according to the drawing of Figure 4. These could be charged with 2–4 kilograms of leaf meal.

[22] Unpublished.

The method varies somewhat in practice according to the chlorophyll content and the fineness of the leaf meal. Since the extraction progresses downward in sharply defined layers and each layer is immediately exhausted, we investigate, by probing with a spatula, to what depth the extraction has proceeded and the material has been decolorized, that is, has become yellowish or gray.

The chlorophyll-rich stinging nettle leaves, collected by ourselves, require much more solvent than commercial meals; larger charges of these can be managed.

With small quantities, work is carried out in the same manner as in the above quoted quantitative experiments, for example, one half of a kilogram of good material is extracted with 1.5 to 1.6 l. of solvent in 30 minutes, whereby 0.9 l. of extract, which contains 4.25 to 4.50 g. of chlorophyll, is obtained. Meal of leaves rich in chlorophyll is not used upon the stone suction filter in larger charges than 2 kg. This charge, which is drawn compactly onto the suction filter by means of a machine-produced vacuum, is extracted with 6 l. of 78–80 per cent

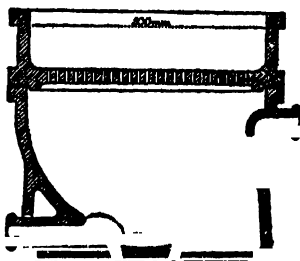


Fig. 4.

acetone which is poured on in several portions, alternately pouring some on, letting it sink in and then sucking it through. The duration of the experiment is 30–45 minutes. The quantity of extract amounts to 4 l. with a yield of 16–17 g. of chlorophyll. The meal becomes straw yellow in color and the lower layers are still greenish only in a few places.

Four kilograms of commercial stinging nettle meal make a charge. This requires, for example, 6–8 l. of 90 per cent alcohol which is filtered in 20–30 minutes, that is, more quickly than aqueous acetone, and yields 3.5–5.5 l. of extract containing 19–24 g. of chlorophyll. A worker in a laboratory can prepare, with two suction filters, 12 such

charges daily (compare use for the preparation of pheophytin in section 3 of Chapter XIII).

The chlorophyll is protected from allomerization in the acetone solution; the extracts described consequently furnish preparations whose cleavage products are especially pure. Nor is the chlorophyll much injured in alcoholic extracts, on account of the quickness of their preparation and because of the water content of the alcohol.

Working up Fresh Leaves. Fresh leaves also are best extracted with 80 per cent acetone. A preliminary treatment with aqueous alcohol as a result of which their grinding is much facilitated has been referred to above (section 1) as valuable in the working up of different plants in an undried state. The usual plant material, namely, stinging nettle leaves, can, however, be ground more quickly and more simply, and without other treatment, in a suitable motor-driven syenite roll mill. The material is then subjected to a preliminary extraction with a little acetone which dehydrates it, removes the mucilages and arrests enzyme action. In consequence of the greater volume the consumption of solvents is considerable and the extracts are diluted, but thanks to the preliminary treatment the degree of purity is far higher, for example, 21, while in corresponding extracts of well dried leaves it is usually 8–10.

Two and a half kilograms of fresh nettle leaves are ground three times during the course of a half hour in a mill whose rolls are gradually set more closely together. A deep olive brown sticky mass is thus obtained without damaging the chlorophyll. Since the leaf substance in this condition can be pressed only with difficulty and with loss of chlorophyll it is first shaken for a short time with 1.5 l. of acetone in a wide-mouthed bottle and then filtered on a suction filter; the yellow to brownish fluid contains no chlorophyll and does not color ether. The residue is placed in a filter-cloth and pressed with a Buchner press, using about 200 atmospheres pressure; the press cake weighs, for example, 0.8 kg., 0.5 kg. of which is dry substance. The cake is broken up and ground twice again in the syenite mill. The powder is then shaken for 5 minutes in a bottle with 1.5 l. of acetone, which is diluted by the moisture that has been retained by the leaf substance to 80 per cent by volume and then still another liter of acetone (80 per cent) is added. The solvent action of the admixtures cannot be used in this case to the same extent as in the case of the dry meal and the extraction cannot be carried out simply upon a

suction filter since the solvent would run from the voluminous mass too quickly. The dilute mass is finally subjected to strong suction again upon the suction filter and then washed three times, each time with 0.5 l. of 80 per cent acetone, with alternate maceration and suction. The meal remains almost free from green pigment. The extract, which required one and a half hours in all to be obtained, contains 4.7 g. of chlorophyll in 3.7 l.; in consequence of its water content it is poor in troublesome accompanying substances.

The method proved to be especially valuable in working with brown algae (Fucus). After the first grinding the preliminary extraction with acetone separates a very large quantity of troublesome mucilaginous substances which would make the extraction more difficult, afterwards interfere with the separations, and hinder very much the isolation of the pigment.

IV. QUANTITATIVE ANALYSIS OF THE FOUR CHLOROPLAST PIGMENTS.

Our quantitative analysis of the two green and the two yellow pigments of the chloroplasts is based upon their colorimetric comparison with solutions of known content and is used to determine the quantities and the relative proportions of these pigments in the leaf, in extracts, or in preparations.

We have further developed the principles of procedure of Willstätter and Isler, who have in a fundamental investigation dealt with the determination of the relative proportions of the chlorophyll components *a* and *b*. In addition there is the treatment of the new problem; viz., the determination of the relative quantities of the two yellow pigments with respect to one another and to the green pigments.

The methods that serve for the quantitative determination of the chlorophyll mixture are first given and those methods follow that deal with the relative proportions of the green and the yellow pigments.

1. Determination of Chlorophyll (a + b).[1]

The crude chlorophyll solution contains the four pigments, which differ in color and color intensities, in relative proportions that are influenced by the solvent, and which may be dependent upon the species of the plant and even upon the harvest of any particular plant. The relative proportions of the components in the extracts of a given plant are approximately the same; therefore a relative determination of its chlorophyll content may be made by a comparison of these. For the investigation of extracts of dissimilar color tint, as for example, from different plants, it is necessary to separate the pigments by saponification with alkali into the indifferent yellows and the alkali salts of the green pigments. The chlorophyllin solutions thus obtained make possible the relative determination of the color value, and furthermore, their comparison with an alcoholic solution of known chlorophyll content permits the absolute determination of the pigment content.

[1] See also Ann. d. Chem. 371: 11, 1909 and 380: 177, 1911.

The proportion of the blue-green component of chlorophyll to the yellow-green component likewise varies in extracts and in preparations. The comparison-preparation of chlorophyll or pheophorbide is therefore chosen or mixed so that its component ratio is similar to that of the sample to be examined.

(a) *Relative Determination.*

In order to ascertain what percentage of the chlorophyll that is extractable from a plant material has passed into a solution, this is compared with the quantitative extract from a small, weighed amount of the same material. In this way the colorimetric comparison shows no, or only insignificant, differences in hue.

For example, in addition to the large scale elaboration of a leaf meal a small percolator is charged with 100 or 200 g. of the same powder and exhaustively percolated with alcohol (*i.e.,* till the run-off is colorless) or 10–50 g. of leaf meal are quantitatively extracted upon a small suction filter with 85 per cent acetone. With meal not very finely ground this is accomplished in an hour. For the colorimetric comparison, the extracts are most advantageously diluted with the solvent so that 1 kg. of leaf powder yields 200 l. of the extract.

The percolate from 36 kilograms of stinging nettle (mixed with the second runnings of a previous charge) contained 79.3 per cent and the second runnings of this percolate 10.9 per cent of the total chlorophyll. The percolate from a charge of only 3 kg. stinging nettle contained 78.8 per cent of the possible yield.

In the same manner, by the aid of quantitative extracts of weighed quantities of leaves a comparison may be made of different supplies or harvests of the same plant, of fresh and dried leaves of an individual plant, and also of leaves from different plants.

Each 10 g. of meal of dried stinging nettles, whether collected by us or commercial, was exhaustively extracted upon a small "Nutsch" in the course of an hour with 200 cc. of 85 per cent acetone. The extract was diluted to 200 cc. with the same solvent and 10 cc. of this solution were diluted with 95 per cent alcohol to 100 cc. A colorimetric comparison of these solutions showed that the meal from leaves collected by us contained 1.71 times as much chlorophyll as the commercial stinging nettle meal. The quantities of chlorophyll in the quantitative extracts were also determined absolutely by comparison with a

solution of known chlorophyll content. The values found showed that the ratio of the chlorophyll in the leaves collected by ourselves to the chlorophyll in the technical material was as 1.70 : 1.

The relative determination also makes possible an absolute determination of the chlorophyll content of solutions after the chlorophyll content of a leaf meal has been once determined absolutely.

(*b*) *Absolute Determination.*

The quantitative determination is carried out by comparison with pure chlorophyll. Formerly, only the so-called "crystalline chlorophyll" (ethyl chlorophyll mixture) was available for this purpose. Comparison with this ethyl chlorophyllide made control of the increase of purity possible in the isolation of the natural phytyl chlorophyllide mixture, and finally the production of the pure preparation. To-day, both chlorophyll mixtures are equally easily obtainable in the pure state and both are used for the preparation of colorimetric solutions for comparison.

A suitable concentration of normal chlorophyll solution is obtained with 0.0500 g. phytyl chlorophyllide or 0.0362 g. ethyl chlorophyllide[2] in 2 l. of alcohol.

Therefore 0.0500 g. chlorophyll, increased by 2.5 per cent to compensate for loss in drying, *i.e.*, practically 0.0513 g., or 0.0362 g. ethyl chlorophyllide with 5 per cent extra loss in drying (100°, high vacuum), *i.e.*, 0.0380 g. is dissolved in 100 cc. of absolute alcohol; this solution can be stored in the dark. For colorimetric comparison 10 cc. of this solution are diluted to 200 cc. The solution to be tested is brought to approximately the same concentration.

Agreement in hues is brought about by the choice of a comparison preparation with a component ratio similar to that of the material which is being tested, or the isolated components *a* and *b* are mixed in that ratio.

The quantitative separation of the green pigments from the yellow pigments which would influence the intensity and the hue is required for the evaluation of a crude solution or a crude product. A small, measured test portion (10 cc.), for example, of an alcohol or acetone extract, is diluted to 100 cc. with ether; 10 cc. of the mixture are placed in a separatory funnel and diluted further with ether, about 5

[2] The calculation is here based upon the mol. wt. of the *a* component.

times. In the case of an alcoholic extract, the dilute ethereal solution can be vigorously shaken at once with 4–5 cc. of methyl alcoholic potash, whereupon the brown phase appears; but if the solution contains acetone, this must be completely washed out of the ether before the saponification, because alkaline chlorophyllin is damaged by acetone. This is done by adding some methyl alcohol and washing with water. After the return of the pure green color, water is slowly poured into the separatory funnel while shaking and the xanthophyll, which is first taken up by the methyl alcoholic lye solution, is again given up to the ether. The alkaline, aqueous chlorophyllin solution is run into a 200 cc. graduated flask, the ethereal solution of the yellow pigments is shaken again with water and the pure green solution is diluted with alcohol to 200 cc. The chlorophyllin solution is now compared in a Dubosque colorimeter with the above described solution of known content. By interchanging the receptacles several readings are made and their averages determined.

The comparison of the saponified chlorophyll with the unsaponified chlorophyll of the standard solution involves no error; 10 cc. of an ethereal solution of pure chlorophyll (0.0500 g. in 100 cc.) were diluted to 200 cc. with alcohol, and another 10 cc. were saponified with 3 cc. of methyl alcoholic potash, taken up with a little water and similarly brought to 200 cc. with alcohol. The two solutions then agreed well in intensity except that the hue of the potassium chlorophyllin was somewhat bluer.

Examples: 2 kg. of meal with a 7 per cent moisture content, from stinging nettle leaves collected by us, gave 4.35 l. of extract when quickly extracted with 6 l. of 90 per cent alcohol on a "Nutsch," 50 cm. in diameter. A 10 cc. test sample of the extract was diluted with ether to 100 cc., and from a tenth part of this, 200 cc. of chlorophyllin solution was obtained and its chlorophyll estimated by means of the standard solution. The averages of the colorimeter readings were 50 mm. for the test solution and 35.5 mm. for the standard solution. Consequently the extract contained $\frac{4.35 \times 35.5 \times 5}{50} = 15.5$ g. of chlorophyll; that is, 7.75 g. per kilogram of meal.

A quantitative "Nutsch" extract (200 cc.) with 85 per cent acetone from 10 g. of the same meal gave from a 10 cc. portion without subdivision 200 cc. of chlorophyllin solution. The depth of the standard solution in the colorimeter was 43 mm. The chlorophyll content of

the 10 g. sample was therefore 0.086 g.; that is, 8.6 g. in 1 kg. of leaf meal. Hence, there was a chlorophyll yield of 90 per cent in the case of the extract that was obtained on a large scale.

For the estimation of the chlorophyll content of fresh leaves a weighed amount is thoroughly ground with quartz sand and then exhaustively extracted on a "Nutsch" with approximately 90 per cent acetone. A test sample is transferred to ether, the acetone is thoroughly washed out and the yellow accompanying pigments are separated from the green pigments by saponification of the chlorophyll.

The degree of purity of chlorophyll preparations that are free or almost free from yellow pigments, as well as that of preparations of chlorophyllin salts, is determined by the comparison of a weighed amount of the substance in alcohol with our solution of known chlorophyll content. For crude chlorophyll preparations that contain much yellow pigment as impurities the saponification method is employed.

Yields of 6.5–7 g. of crude chlorophyll per kilogram of meal were obtained from good stinging nettle material. Here the ratios of the colorimeter readings were as 50 : 45 to 47.5. The preparations were, therefore, 90–95 per cent chlorophyll.

Preparations from commercial stinging nettle leaf meal required 40–42 mm. of the standard solution to match the color; they were contaminated with 16–20 per cent of colorless accompanying materials.

The quantitative determination of chlorophyll guided the first experiments on the isolation of chlorophyll and, even to-day, is frequently used, when obtaining chlorophyll preparations, for ascertaining the degree of purity of the solutions. After each purifying operation the dry residue is determined by evaporating a sample in vacuo at 100° C. and the absolute chlorophyll content of the solution is also determined.

A crude extract with 85 per cent acetone for the preparation of pure chlorophyll contained 16.8 g. of chlorophyll and 135.2 g. of residue, non-volatile at 100° C. in a vacuum; it, therefore, contained 12 per cent chlorophyll. After the subsequent operations of purification, the degree of purity increased to about 45 and then to 65 per cent.

The difference of color in the two components of pheophytin is even greater than with chlorophyll; *a* is olive green; *b*, red brown. It is therefore still more important with pheophytin to adjust the standard solution to the pheophytin preparation that is under investigation,

the component ratio of which is evaluated by a cleavage test, by mixing the homogeneous methyl pheophorbides or pheophytin components.

The usual comparison solution contains 0.0500 g. of pheophytin of the component ratio 2.5, or 0.0350 g. of methyl pheophorbide; namely, 0.0250 g. of $a + 0.0100$ g. of *b*, dissolved in 25 cc. of chloroform, diluted to 200 cc. with ether, 10 cc. of which is again diluted tenfold.

2. The Relation between the Chlorophyll Components a and b.

(a) *The History of the Method.*[3]

Since the chlorophyll from any plant can be successfully transformed, without the formation of secondary products, into phytochlorin *e* and phytorhodin *g*, the ratio of the components is ascertained by a quantitative determination of the yields of these two compounds in the decomposition. This can be carried out most simply and with very small quantities by a colorimetric comparison of the solutions, that are obtained as a result of the cleavage, with known pure substances.

In the case of the component ratio in isolated preparations, in chlorophyll or in pheophytin, the determination depends chiefly upon quantitatively carrying out the hydrolysis directly to the two normal cleavage products and upon the quantitative separation of the two.

In order to adapt the method to the investigation of the pigment of green leaves, it is required as a further condition that the errors that are possible in extraction shall be avoided.

In their first observations on the component ratio Willstätter and Isler found many pheophytin preparations to be identical. Some individual preparations, however, differed considerably and the pheophytins from Pinus and Melissa, especially, were much richer in the *b* component. It was by means of these apparent exceptions that the chief errors in the method were discovered; namely,

1. A fractionation of the mixture of the two components can take place even in the extraction of the chlorophyll from the leaves.

It is, therefore, necessary to extract the pigment quantitatively if the determination of the pigment in the leaf is to be of value.

2. Pheophytin separates more or less incompletely according to the dilution of the extract, its water content, and according to the amount of accompanying materials. It has been found that the precipitated

[3] Ann. d. Chem. 380: 159, 1911 and 390: 290, 1912.

and the dissolved portions differ in their component ratios. The dissolved part is richer in component *a*.

It is therefore necessary to saponify the pigment quantitatively as pheophytin if a determination of the pigment in the extract is to be of value.

3. Also, in the fractional precipitation of pheophytin; for example, from chloroform with alcohol, there results a displacement of the component ratio in like manner to that in the case of the separation cited above. We, therefore, abandoned the weighing of pheophytin and its purification.

The component ratio of the isolated pheophytin is of interest for the estimation of the given preparations, but it does not express the component ratio of the chlorophyll.

Only when the yield of a certain pheophytin amounts to very nearly the theoretical must its component ratio approach that of chlorophyll. The values found in the case of the pheophytin preparations that were produced by us on a large scale are, therefore, approximately correct.

Willstätter and Isler, therefore, base their method for the determination of the component ratio of the chlorophyll of different plants upon the complete extraction of the pigment, its conversion into crude pheophytin without any loss, as smooth as possible a saponification of this, and the quantitative isolation of the chlorin and rhodin.

By means of this method, Willstätter and Isler commenced determining the ratio of the chlorophyll components for a large number of plants and found that it is, in general, approximately constant; namely, the average of 24 experiments = 2.5(7). The greatest deviations were ± 0.4–0.5 The question was still undecided as to whether these were within the limits of error of the method or not. We now know that these deviations are undoubtedly not merely errors of determination, although the accuracy of the determination was still capable of improvement.

The method also offers a new means of quantitatively determining chlorophyll in extracts and in plants.

Willstätter and Isler in this way find that the chlorophyll content of leaves is in most cases 0.7–1 per cent of the dry weight.

The results in regard to the composition of chlorophyll with respect to its two components differ greatly from former asssumptions.

H. C. Sorby[4] estimated the ratio spectrophotometrically, under the incorrect assumption that there is an equal absorptive power by the

[4] Proc. Roy. Soc. 21: 480, 1873.

two components in the red, by diluting the chlorophyll solution in a tube to such an extent that the first absorption band of component *a* appeared of the same intensity as the first band of component *b* in a second tube. His determinations gave, in the case of sound green leaves, 5.9 to 7.7 parts of chlorophyll *a* to 1 part of chlorophyll *b*.

Regarding the variability of the ratio Sorby decided: "The normal relative amount of yellow chlorophyll in green leaves certainly varies, and there seems reason to believe that this to some extent, if not mainly, depends on the length of time to which they have been exposed to the sun."

Tswett[5] obtained similar values by a spectroscopical estimation and, especially in connection with his chromatographic adsorption analysis, by a comparison of the heights of the chlorophyll zones *a* and *b*, which appear in their color to be approximately similarly saturated. His determinations give the ratio a : b = 4 to 6 : 1.

Lately, C. A. Jacobson and L. Marchlewski[6] have also published an investigation, "On the duality of chlorophyll and the changing ratio of its components." They find that climatic conditions appear to play an important rôle in the production of one or the other component of chlorophyll and they give in addition several proofs for the proposition that the ratio of the chlorophyll components varies with the plant species and with the conditions of growth of the same species.

The principal errors of this investigation are that: only an optional part of the chlorophyll in a plant was extracted for the determination of the component ratio; an optional unknown part of the extracted pigment was precipitated as pheophytin and an optional part of the crude pheophytin was isolated by fractional separation.

These main errors of the principles of the method were surmounted by Willstätter and Isler.

Upon the basis of their investigation we extended our work and found no essential errors or imperfections but learned to avoid the inaccuracies in the saponification of pheophytin and in the fractionation of the two cleavage products which exert considerable influence upon the value of the component ratio and upon the judgment of the constancy of the same.

[5] Ber. d. d. bot. Ges. 25: 396, 1907.
[6] Biochem. Zeitschr. 39: 174, 1912.

(b) *Sources of Error in the Determination.*[7]

Incomplete Extraction.

The quantitative extraction of chlorophyll is one condition for ascertaining the true ratio.

In the partial extraction of chlorophyll from fresh or dry leaves the components do not enter the extract in their natural ratio, but there is a fractionation.

For example, we extracted 40 g. of stinging nettle leaf meal with ethyl alcohol, using a suction filter and pump, as thoroughly as possible with this solvent. The extract was consequently so dilute (over 300 cc.) that no pheophytin separated on acidification. A little water was gradually added till a separation of pheophytin resulted. This was filtered off and then quantitatively dissolved from the filter by means of ether; after evaporation the ratio of *a* to *b* was determined in the residue. The alcoholic mother-liquor was extracted quantitatively with ether, and the ratio was also determined in the portion of the pheophytin thus isolated.

	Grams of		Component ratio
	Phyto-chlorin *e*	Phyto-rhodin *g*	
Pheophytin, separated	0.0123	0.0121	1.04
Pheophytin of the mother-liquor, 1st test	0.0173	0.0034	5.22
Pheophytin of the mother-liquor, remainder	0.0215	0.0050	4.38
Pheophytin of the subsequent extraction, 1st test	0.0054	0.0039	1.41
Pheophytin of the subsequent extraction, remainder	0.0081	0.0048	1.73
Total pheophytin	0.0646	0.0292	2.26

Leaf meal that has been extracted on the "Nutsch" still contains, according to previous experience, at least 1/5 of the chlorophyll, which can be extracted only with difficulty.

The meal was shaken in a bottle with considerable alcohol and filtered and this operation was repeated twice more to secure complete exhaustion. These combined subsequent extracts were acidified and then extracted with ether for the determination of the ratio.

[7] Ann. d. Chem. 390: 294, 1912. Partially unpublished.

The "Nutsch" extract which contained 76 per cent of the total chlorophyll consequently gave the quotient 2.55; the subsequent extracts furnished the value 1.57. The portion of the chlorophyll that remains in the leaves is therefore much richer in the *b* component.

It appears at once from this experiment that the pheophytin that is separated from the acidified extract shows an entirely different component ratio than the portion that is dissolved in the mother liquor (in this case almost 2/3); the latter portion is always poor in component *b*.

Incomplete Separation.

A great number of experiments, which have been carried out with different materials, indicate that the most important error involved in testing the isolated pheophytin instead of the total extractable pigment lies in incomplete separation.

	Grams of		Component ratio
	Phytochlorin *e*	Phytorhodin *g*	
Separated pheophytin	1. 0.0049	0.0054	0.93
	2. 0.0046	0.0050	0.94
Pheophytin from the mother-liquor	1. 0.0095	0.0038	2.58
	2. 0.0082	0.0037	2.27

For example, 100 g. of commercial stinging nettle leaf meal was exhaustively extracted on a "Nutsch" with ethyl alcohol and the dilute extract was treated with oxalic acid. The pheophytin that separated after two days' standing was determined in aliquot portions by saponification and fractionation. On the other hand, for purposes of comparison, the remainder of the pheophytin (57–58 per cent of the total quantity) was extracted with ether from the alcoholic mother liquor.

In the same way as by dilution the quotient is shifted unfavorably to the component *a* by the use of more concentrated alcohol, for example, a percolation experiment with 99 per cent alcohol gave a pheophytin preparation with the ratio 1.38 while an extract of the same chlorophyll concentration, obtained with 95 per cent alcohol, furnished pheophytin with a quotient of more than two.

Fractionation by the solvent could have had little influence upon the composition of our pheophytin which was obtained in large quan-

tities and with good yields. For if the portion that precipitates spontaneously amounts to about 4/5, the quotient of the preparation will not deviate too much from the true component ratio of the chlorophyll extract even though a pheophytin fraction that is rich in the component *a* remains in the mother-liquor.

It is quite otherwise in the case of the isolation of pheophytin from leaves that contain very much of extractive materials and are consequently little suited for the preparation of pheophytin. Pheophytin separates spontaneously in only small quantities from the acidified extracts of certain plants since the colorless accompanying substances keep it dissolved. This solvent action is selective for the components; indeed much more so than that by alcohol alone.

There are consequently obtained in the case of the spontaneous and incomplete separation of pheophytin in certain instances, for example, from Pinus and Melissa, preparations whose cleavage gives abnormally high yields of phytorhodin. The composition of the chlorophyll in extracts or in leaves may not be judged, therefore, by the composition of the pheophytin preparation that has been precipitated to an arbitrary extent.

Examples: Willstätter and Isler placed 800 g. of fresh spruce needles (Picea excelsa) (March) in a mixture of 1,500 cc. methyl alcohol, 600 cc. ether and 900 cc. water, according to their method of preliminary treatment, and allowed them to remain immersed three hours. The needles were then centrifuged, ground in a syenite mill, and shaken in a bottle with alcohol. The extract was filtered off by suction and oxalic acid added to it. The small amount of pheophytin (0.3 g.) that separated was filtered and subjected to fractional precipitation.

Pheophytin used 0.0616 g.
Phytochlorin *e* found 0.0141 g.; that is, 22.9% of the pheophytin.
Phytorhodin *g* found 0.0222 g.; that is, 36.0% of the pheophytin.
Total 0.0363 g. of cleavage products; that is, 58.9% of the pheophytin.

The component ratio according to this is, 0.65; the pheophytin is therefore extraordinarily rich in the *b* component; three fifths of it consists of *b*.

In order to determine the component ratio correctly in a parallel test of the spruce needles, they were dried in the air and their quan-

titative extract, without precipitating the pheophytin, was converted to chlorin and rhodin.

Ten grams of the meal yielded 0.0118 g. of phytochlorin *e* and 0.0038 g. of phytorhodin *g*. Component ratio 3.19.

Melissa meal (like Sambucus) produced, upon percolation and spontaneous separation of the pheophytin, preparations with the component ratios 1.01 and 1.48. The yields of pheophytin were 1.5 and 2.1 g. per kilogram.

On the other hand, the same meals gave the ratio 2.5 according to a determination based upon the quantitative isolation of pheophytin.

Incomplete Fractional Precipitation.

In addition to the influence of the solvent in the first separation of the pheophytin there is another, considerably less important error, namely, an additional fractionation when the pheophytin is fractionally precipitated; *e.g.*, from chloroform by means of alcohol.

If the crude pheophytin, containing oxalate, is saponified, there can be no control of the determination by means of the sum of the cleavage products, which should amount to close to two-thirds of the weight of the pheophytin. But if the pheophytin is fractionally precipitated then the precipitated portion is again somewhat richer in component *b*.

This displacement was shown very clearly on treatment with alcohol. A useful method for the isolation of either one of the components can by no means be obtained by their fractional separation from solvents because the considerable differences in solubilities of the pure pheophytin components are not sufficiently pronounced in their mixtures. The two components influence each other mutually in their solubility.

Example. 2.5 g. of pheophytin (I) rich in component *b*, from Sambucus, were dissolved in 1,700 cc. absolute alcohol at boiling temperature; after cooling, 0.8 g. (II) separated, over night, as a granular micro-crystalline deposit. The deposit was dissolved a second time in 550 cc. absolute alcohol; from this only a very little separated (III) and the filtrate, after standing several days, furnished 0.2 g. more of pheophytin (IV).

The component ratio of I is 1 1/3; II is 2/3; III is 1/4; IV is 1/3 to 2/5.

Errors in Saponification and Fractionation.

Chlorophyll, as such or in the form of its magnesium free derivative, cannot be separated into its two components without a loss. The

very feebly basic pheophytins require for their separation hydrochloric acid of such high concentration that the phytol ester group is easily attacked. Besides, the strong acid would cause troublesome emulsions with the crude pheophytin solution. The separation is therefore carried out with the products of the alkaline hydrolysis of the pheophytin since they are much more strongly basic (hydrochloric acid numbers 3 and 9).

But the formation of these compounds is no simple process; it takes place through the hydrolysis of the easily saponified group α: $COOC_{20}H_{39}$ and of the difficulty saponifiable group β: $COOCH_3$ and with simultaneous relactamization. In this process a lactam group of the pheophytin is broken in the brown phase and a new one is formed. It is difficult to direct this process quantitatively in one direction. Even under the most favorable conditions, as in working up the pure chlorophyllides and pheophorbides, there is always the danger that relactamization may take place to a minor extent in a second direction and that, in consequence, the cleavage products that are characterized as normal may be admixed with the weakly basic phytochlorin *g* (hydrochloric acid number 11) and phytorhodins *k* and *i* (hydrochloric acid numbers about 15).

The best conditions for saponification are: hot and rapid; if a dilute cold solution is used, much weakly basic material is formed.

Chlorophyll and chlorophyllide, as such, can be saponified and converted into chlorin *e* plus rhodin *g*, without secondary products, by introducing them into concentrated methyl alcoholic potash while boiling. But weakly basic cleavage products become detectable upon even very slight dilution of the chlorophyllides; *e.g.*, with some pyridine.

Pheophytin itself cannot be converted quantitatively into chlorin *e* and rhodin *g* if it is introduced undissolved into the boiling lye. Some rhodin is destroyed even in the time required for its complete solution.[8] Hot saponification in a concentrated pyridine solution is more favorable; the loss of rhodin is avoided but a couple per cent of weakly basic derivatives are unavoidable in this case.

[8]For a time it was considered, when cold saponification was compared with hot saponification, that the latter, on account of the sensitiveness of the phytorhodin, was the more unfavorable (Ann. d. chem. 380: 161, 1911). The time involved in the saponification was too long. If the time of the hot saponification is sufficiently shortened, it is the superior method and it is particularly so since the time when the use of pyridine was introduced.

In the necessity of analyzing all the chlorophyll of an extract there lies the disadvantage that it cannot be obtained in a pure form but that its magnesium-free derivative, together with a considerable amount of impurities, is isolated and saponified. These impurities are themselves, for the most part, saponifiable; they make complete hydrolysis difficult, even under energetic conditions.

Willstätter and Isler essentially surmounted this difficulty by carrying out the cleavage of the crude pheophytin in pyridine with considerable methyl alcoholic potash and short boiling. The fact was not overlooked that, even under the conditions chosen, the course of the reaction did not yet run entirely smoothly and that analytical errors could be responsible for considerable variations in the component ratio found. This found expression in the following statement:

"The ratio numbers of all our determinations deviated from the average value, 2.57, by ± 0.4–0.5, with an average deviation of ± 0.23. These deviations of about 20 per cent from the mean probably lie within the range of possible errors. It is necessary to still further improve the method in order to use it for the testing of smaller differences in the composition of chlorophyll."

The uncertainty is conditioned first and chiefly by the variable amount of the feebly basic secondary products. In preparative work the feebly basic products are infinitesimally small in amount, but in analysis even a few per cent cause disturbing fluctuations.

The pure methyl pheophorbides (3 parts of *a* and 1 part of *b*) were saponified separately under the same conditions as prevail in the process of analysis. After the extraction with 3 per cent hydrochloric acid there still remained unextracted one-tenth of the phytochlorins which would in the determination join the rhodin, increase this by about 25–30 per cent. On the other hand, in the 12 per cent hydrochloric acid rhodin extract, 10 per cent of the feebly basic rhodin was lacking; the error was diminished by that much. Instead of a true component ratio of 3, 2.25 would be found in this case. Naturally such errors are not uniform and are not always so large as in the case quoted.

The disturbing influence of the feebly basic secondary products has been reduced to a minimum by improving the hydrolysis of the pheophytin, namely, by still more energetic conditions for saponification.

A second error in the determination is the inexactness of the separation of phytochlorin and phytorhodin with 3 per cent hydrochloric

acid when the fractionation—differently than in making preparations—must be done without washing on account of the otherwise unavoidable loss of material. Some of the phytorhodin then always passes over into the extracts of the stronger base. In the example which has been cited for methyl pheophorbide *b*, 5 per cent of the rhodin was lost in an extraction with 3 per cent hydrochloric acid as would be done in working up a mixture.

This fractionation error is found in all the analyses by Willstätter and Isler; it partially compensates the uniformly occurring error involved in saponification. Consequently, in our example, the component ratio was found to be, instead of 3, not 2.25 but 2.4.

On the whole, as a consequence of these inaccuracies, the values found by Willstätter and Isler for the component ratio, may have been 20 per cent too low.

These difficulties may be successfully overcome if solutions of phytochlorin and phytorhodin are used as comparison materials instead of weighed amounts of them; these solutions are obtained from a mixture of weighed amounts of the methyl pheophorbides *a* and *b* in the course of analytical separation.

(c) *Characteristic Features of the Method.*[9]

The principle of the earlier method is retained; decomposition of the chlorophyll to phytochlorin *e* and phytorhodin *g*, separation with hydrochloric acid and colorimetric determination.

In order to ascertain the component ratio in finished preparations of chlorophyll or chlorophyllides, alkaline hydrolysis, which proceeds smoothly only with the solid material, is carried out. The isochlorophyllin salts, which are the magnesium compounds of the two normal cleavage products that are formed, are decomposed by acidification.

In the determination of the dilute chlorophyll of a plant extract, cleavage proceeds best by way of pheophytin. Instead of acidifying the solution of chlorophyll as formerly and extracting the pheophytin, the chlorophyll, which is more easily soluble in ether, is extracted with this solvent before it is subsequently decomposed with acid.

The saponification of the crude pheophytin is carried out by boiling its pyridine solution for several minutes with considerable, highly concentrated, methyl alcoholic potash with the addition of water; this saponification takes place so smoothly that the quantity of weakly basic

[9] Unpublished.

by-products amounts to only 2–3 per cent. In all cases, the weakly basic rhodin remaining after the fractionation is compared colorimetrically in 17 per cent hydrochloric acid with the main solution of rhodin *g*. The analysis is of use only when the yield remains under 3 per cent, which is almost always the case.

The most important improvement of our method as compared with the one that has been published consists in the choice of the materials for comparison, which eliminates the still unavoidable errors of saponification and fractionation. The materials for comparison consist of the methyl pheophorbides *a* and *b* in the approximate ratio of the mixture to be investigated; namely, 1 mole of methyl pheophorbide *b* to 3 moles of *a*. The same saponification and fractionation is carried out in parallel with these comparison preparations so that the same errors occur in the comparison solution and in the one under investigation. In addition to this, the methyl pheophorbides offer the advantage that they can be easily prepared pure. They are stable and undergo no loss on drying.

In order to test the accuracy of the method, almost all the determinations were carried out simultaneously in duplicate.

In order to determine the yellow chloroplast pigments in addition to the green ones and the ratio of carotin to xanthophyll, as well as the molecular ratio of the yellow to the green pigments, double the quantity of leaves is extracted and the ethereal chlorophyll solution is divided in half. In one portion the chlorophyll is saponified by lye and separated. For the quantitative separation of the two carotinoids, a method has been worked out which is also suitable for purposes of preparation. It is free from error and consists in a distribution between petroleum ether and aqueous methyl alcohol. The carotin remains in the petroleum ether phase while the xanthophyll goes into the methyl alcohol.

For comparison the two pure pigments are dissolved in the same solvents.

Since the method for ascertaining the ratio of the pigments in the plant provides for their quantitative extraction, it gives also the amounts, by weight, of the individual pigments in the leaves.

Colorimetric and spectroscopic methods were available for the determinations.

Preference was given to the former.

The colorimetric determination necessitated the perfection of the method of separation of the pigments and of the reactions employed in decomposition; analytical work in this field is closely associated with technical treatment; it is adapted to preparative purposes.

The complicated composition of the mixture of natural pigments in which the preponderant component *a* optically masks the other constituents and, furthermore, the instability of the pigments, whose absorption ratios may be altered considerably even by the influence of a small quantity of plant acid in the extract, oppose the use of spectrophotometric methods.

A further reason for preferring the less accurate method of determination lay in the fact that the difficulties and errors in the working up of plants, for example, in the extraction of the pigments, are so important that a measurement of great exactness is worthless until fundamental methods exist for the isolation of the pigment. After these are worked out, the methods of quantitative spectral analysis can be drawn upon in the future for the improvement of the determination.

(d) *Analysis of Chlorophyll Preparations*[10]

The component ratio and the degree of purity of crude chlorophyll and of pure preparations of chlorophyll and crystallized chlorophyllides are ascertained.

5–6 cc. of boiling 35 per cent methyl alcoholic potash are poured over 0.050 grams of the dry powdered material; the test-tube has been previously warmed to 60° and the lye is immediately brought to a boil again and kept boiling gently, without evaporation of the methyl alcohol, till the chlorophyll dissolves to a clear solution. Heating is continued 15 seconds longer and, after cooling, the lye is washed with water into a 500 cc. separatory funnel. The solution is now acidified rather strongly with 20 per cent hydrochloric acid in order to decompose the complex compounds that may have been formed by the solution of zinc from the glass. It is then vigorously shaken with 250 cc. of ether and the acid is neutralized with ammonia till the aqueous layer is only very pale blue. The last traces of chlorin are only completely extracted with 50 cc. more of ether. The two ether extracts are combined and the methyl alcohol, which would influence the hue of the

[10] Unpublished.

hydrochloric acid chlorin extract, is removed by washing twice with 200 cc. of water each time.

The ether is extracted 3 times, each time with 120 cc. of 3 per cent hydrochloric acid, then twice, each time with 50 cc. of 5 per cent acid. The two last extracts must be fractionated again. The bases are transferred again from these last two extracts into 30–50 cc. of ether and the chlorin is extracted again 2–3 times with 3 per cent acid. The remaining, feebly red-colored ether becomes the main phytorhodin solution. This is now extracted with 12 per cent hydrochloric acid; *e.g.*, three times, using 120 cc. each time. Depending upon the nature of the preparation, the ether that remains is colorless or yellow; 20 per cent hydrochloric acid should extract only a trace of pigment, or none at all, from it.

The *a* component is now present in the form of chlorin *e* in the ether-saturated, 3 per cent hydrochloric acid, and *b* as rhodin *g* in the 12 per cent acid; both extracts are brought to volumes of 500 cc. with ether-saturated hydrochloric acid of corresponding concentrations.

Comparison Solutions. The component ratio in the preparation under investigation is estimated by means of the cleavage test; for pure chlorophyll it is customarily 2.5. In this case, 0.0356 g. of chlorophyll *a* and 0.0144 g. of chlorophyll *b* are mixed and the mixture is saponified and fractionated as described so that the volumes of the comparison solution and the test solution are the same.

The procedure in this preparation of comparison solutions has been proved to be faultless—with magnesium compounds the saponification proceeds without the formation of secondary products, which is different than in the case with pheophytin—by testing the rhodin solution colorimetrically with the corresponding quantity of weighed pure rhodin. 0.0096 g., with an additional 0.4 mg. corresponding to the loss on drying, was dissolved in a little ammonia and, by acidification, transferred to 300 cc. of ether. In order to work in exactly the same manner as in the fractionation of a mixture, the ethereal solution was first shaken with 500 cc. of 3 per cent hydrochloric acid, which took up a trace of material, and it was then extracted with 12 per cent hydrochloric acid. The solution of phytochlorin thus obtained agreed with our comparison solution for *b*, the difference being only 1 per cent in favor of the latter.

Example 1. Pure chlorophyll, produced from dry leaves to the extent of 6.5 grams per kg. of leaf meal.

Colorimeter depth of the comparison solution: 50 mm.
" " " " test " *a*: 51.5 mm; amount of a = 0.0342 g.
" " " " " " *b*: 49.5 mm; amount of b = 0.0146 g.

Component ratio $= \frac{2.5 \times 49.5}{51.5} = 2.40$; degree of purity 98.

Example 2. Pure chlorophyll, prepared from fresh leaves in the amount of 1.6 g. per kg.

Colorimeter depth of the comparison solution: 50 mm.
" " " " test " *a*: 50 mm; a = 0.0356 g.
" " " " " " *b*: 56.5 mm; b = 0.0127 g.
Component ratio = 2.82; degree of purity 97.

For the determination of the components in pheophytin we proceed in an analogous manner, using the separated pheophytins or methyl pheophorbides as comparison substances; the saponification of the magnesium-free compounds is carried out in concentrated pyridine solutions.

3. Determination of the Four Leaf Pigments.[11]

Extraction.

We collected the leaves for all our determinations, keeping in mind the special growing conditions; the season and time of day, the weather, side exposed to the sun or in the shade, and the position on the plant. The same sample of leaves was always used for duplicate experiments and usually the leaves themselves were cut in half.

Willstätter and Isler carried out most of their experiments with dried leaves; that is, with 10 gram samples corresponding to a chlorophyll content of 0.05–0.10 g. The freshly picked leaves were separated from their stems and midribs and dried, in quantities of 50–100 g., over sulphuric acid in a vacuum. The leaves, having become brittle, were comminuted with a cutting machine and converted to a coarse powder by means of a Universal mill; finally, this coarse powder was ground to the fineness of dust in a porcelain ball mill.

The extraction, in the work quoted, was carried out by the use of ethyl alcohol in very small percolators placed upon suction flasks. The new "Nutsch" method, using the aqueous solvent, is simpler and more

[11] Unpublished.

certain. Ten grams of leaf meal were exhaustively extracted upon a suction funnel, at first with 85 and then with 90 per cent acetone. This required about an hour and gave 200–300 cc. of extract.

In the new series of experiments reported here the leaves were always extracted while fresh. They were worked up in parallel experiments immediately after picking and in all cases 40 grams were weighed out at the same time for the moisture determination. This sample was dried to constant weight, in a vacuum desiccator over sulphuric acid, in 24 to 48 hours.

Previous to the extraction the leaves are treated with aqueous acetone which softens them and removes colorless extractive matter, for example, plant acids, from them, while no trace of chlorophyll is dissolved by it. Disturbing enzyme reactions, for example, oxidase effects, are checked in this way so that the leaf meals are finally left almost colorless, or colored a faint gray only, while without the preliminary treatment they become dark yellow to brown.

For each individual experiment 40 grams of leaves are covered in a large, unglazed mortar, of about 25 cm. diameter, with 50 cc. of 40 per cent acetone, and quickly pulverized with 100 grams of quartz sand. This facilitates not only the comminution but serves also, in the extraction, as a diluting medium for the somewhat mucilaginous leaf material. After the disintegration, if no larger leaf constituents, except the chlorophyll-free parts of veins, are distinguishable, the rather dry pulp is again covered with 100 cc. of 30 per cent acetone and filtered, after stirring for a short time, upon the "Nutsch" through a thin layer of talc, which retains even the fine particles of protoplasm. Then it is washed with 30 per cent acetone also; for example, with 100 to 200 cc., till the filtrate, which at first is frequently brown (in the case of horse chestnut, poplar, and beech), runs off colorless. The comminution and preliminary extraction require 15 to 30 minutes.

The aqueous acetone is well removed by suction and the mixture of leaf material and sand is macerated for several minutes with pure acetone while being loosened up with a spatula, and again subjected to thorough filtration under suction. Complete extraction, with repetitions of the maceration, requires, according to the condition of the leaves and the fineness of the particles, 400–600 cc. of acetone which, toward the end, is mixed with 5–10 per cent of water. After the extraction the acetone, even in the case of lengthy action upon the powder, runs off colorless from the "Nutsch" and even the coarser leaf constituents are decolorized.

The pure green, acetone extract is poured, in portions of 100–200 cc. as they are obtained during the process of the extraction, into 200–250 cc. of ether and the greater portion of the acetone is washed out with distilled water. The removal of the acetone is completed, when all the chlorophyll has been united, by allowing water to flow down along the wall of the separatory funnel during careful rotation and, finally, without any rotation; emulsions were thus avoided. Finally, the ether is dried with some sodium sulfate and filtered into a 200 cc. volumetric flask, which is filled to the mark with ether. The solution of pigments, halved, then serves for the determination of the green and the yellow components.

Separation of the Chlorophyll Components.

100 cc. of the ethereal solution of crude chlorophyll (corresponding to 20 g. of fresh leaves) are washed into a distilling flask (of the type shown in figure 13, Chapter XVII) and 0.5 cc. of $2N$ alcoholic hydrochloric acid is added. The chlorophyll is, by this means, converted into pheophytin which is protected from allomerization by the hydrochloric acid; hence the analysis can be interrupted over night at this stage.

The ether is then evaporated in the cold in a vacuum and, finally, under the very strong suction of a pump for a very short time at 60° C.

The rarely granular, usually waxy, pheophytin, which remains, is dissolved in as little pyridine as possible (1–2 cc.). In a test tube, the rim of which has been broken off, 25–30 cc. of concentrated methyl-alcoholic potash is brought to a boil; the flask with the pyridine solution is then warmed in a boiling water bath and the boiling lye is poured into the flask by introducing the test tube into the neck of the flask and upsetting the glass while simultaneously shaking the whole. The boiling must not cease during this procedure. The brown phase appears and disappears very quickly and the solution becomes olive green. A condenser is placed on the flask and the solution is boiled upon the water bath 2 minutes and then for 1 to 1.5 minutes more, after the addition of 5 cc. of water which is added through the condenser. The flask is now cooled externally under the tap and its contents are washed with water and some ether into a 500 cc. separatory funnel. 20 per cent hydrochloric acid is added till the color changes from a brown to a turbid gray green, 200 cc. of ether are added, and

the funnel is shaken vigorously for several minutes. The turbid aqueous layer, with additions of a little ammonia, is extracted with small portions of ether till the ether is no longer colored. The mother liquor is made alkaline with ammonia, on account of the floccules that are often formed, and is acidified again in order to extract with ether an additional small quantity of cleavage products. As a control the floccules are again covered with dilute ammonia; if but little coloring matter goes into solution, no rhodin has been decomposed by the saponification, whereas, if coloring matter passes into solution, saponification has taken too long a time.

The basic cleavage products require the separation of their accompanying materials before fractionation. The united ethereal solutions are, for this reason, extracted two to three times, each time with 30 cc. of 12 per cent hydrochloric acid, and further, with 10–15 cc. of 20 per cent acid till the acid layer which separates is almost colorless. At the boundary between this layer and the ethereal solution floccules appear again, which, however, no longer contain chlorophyll substance. The ether contains, in addition to the carotinoids, brown pigments, the quantity of which varies with the plant species; strong hydrochloric acid alone dissolves them with a smutty green color.

A layer of 200 cc. of ether is poured over the united acid extracts of the bases in a 0.5 l. separatory funnel and the acid is carefully neutralized with concentrated ammonia, while gently rotating, till the color of the aqueous layer becomes a cloudy blue-violet. The well stoppered separatory funnel, while being cooled under the tap, is then agitated; gently at first and, finally, vigorously. The aqueous layer, which customarily is still pale blue, is allowed to flow into a second separatory funnel. In order to avoid spurting of the ethereal solution, the stopcock is first opened and the lower layer is allowed to flow off till the pressure has become equalized with that externally. With continued neutralization the last traces of the base are transferred into the ether.

The ethereal solution of the cleavage products, from which floccules separate only in case the saponification has not been very good, is washed three times in order to separate the methyl alcohol and some pyridine; this is done each time with 200 cc. of water to which 1–2 cc. of 3 per cent hydrochloric acid has been added since pure water would remove phytochlorin.

We then extract 4–5 times with 3 per cent hydrochloric acid, 400 cc. in all, and then several times with some 5 per cent acid till this is

colored only pale green. The extracts with this stronger acid require additional fractionation. After neutralization they are extracted with 30 cc. of ether and the ethereal solution is repeatedly extracted with 3 per cent hydrochloric acid till the volume of the 3 per cent hydrochloric acid phytochlorin extracts previously obtained is brought to 500 cc. by the addition of these extracts. The ether remaining from the intermediate fraction becomes the nearly pure red, main phytorhodin solution. This is extracted 4–5 times with 12 per cent hydrochloric acid till the volume of the extracts also amounts to 500 cc. The ether that remains is a faint reddish yellow.

Successful saponification and fractionation are confirmed by the insignificant quantity of the weakly basic rhodin, which in a 17–20 per cent hydrochloric acid extract must amount to less than 3 per cent when compared with the 12 per cent hydrochloric acid rhodin solution, and by the insignificant amount of floccules. Results such as these were obtained in almost all tests, so that the values gotten are quoted in the following section without any selection on our part.

Fractionation of Carotin and Xanthophyll.

The second half of the ethereal solution of pigment that was obtained from the 40 g. of fresh leaves is saponified with 2 cc. of concentrated methyl alcoholic potash, with strong agitation, at first by hand and then for 30 minutes in a machine. After standing for some time the ether is usually a pure yellow, but if it still shows a red fluorescence it is shaken for a longer time and a little additional alkali is added if necessary. After complete saponification of the chlorophyll, the ethereal solution is decanted from the potassium salt into a small separatory funnel. A little ether is used, with rotation, to wash the potassium salt. This is not sufficient to extract the xanthophyll; 30 more cc. of ether are added to the sirupy chlorophyllin salt and then, while shaking, water is gradually added, and the time is awaited when the emulsion in the separatory funnel has separated. As a control, the alkaline fluid is thoroughly shaken a second time with ether, which usually remains colorless.

The ethereal solutions are then united and washed with water, to which some methyl alcoholic potash has been added, in order to remove any trace of chlorophyllin and, more frequently, small quantities of brown, acid organic matter, and, finally, twice with pure water. The ether is then evaporated under vacuum at ordinary temperature to a

few cc. in a distilling flask; the residue is transferred to a separatory funnel for fractionation by means of 80 cc. of petroleum ether and the flask is rinsed with a little petroleum ether.

Fractional extractions with 100 cc. of 85 per cent, 100 cc. of 90 per cent and two lots of 50 cc. each of 92 per cent methyl alcohol are made successively for the separation of the yellow pigments. The last extract is usually colorless; if otherwise, the extraction is repeated with 92 per cent methyl alcohol.

The methyl alcoholic xanthophyll extracts are free from carotin; they are mixed with 130 cc. of ether and the pigment is transferred into the ether by the gradual addition of water. This ethereal solution of xanthophyll, and likewise the petroleum ether solution of carotin, is freed from methyl alcohol by washing twice with water; they are run through dry filters into 100 cc. volumetric flasks and a few drops of absolute alcohol are mixed with them, for clarification. Finally the flasks are filled to the mark with ether or petroleum ether.

The Comparison Solutions.

For the Chlorophyll Components. Since the component ratio is not far removed from 3, even in the case of the greatest deviation, the employment of a single mixture of methyl pheophorbides for the preparation of the comparison solutions will cause no great error. This amounts to approximately 3–4 per cent of the ratio number in the most divergent component ratios. The mixture consists of *a* and *b* in the ratio of 3 moles to 1 mole, or

0.0369 g. methyl pheophorbide *a*,
half hydrate, $C_{36}H_{39}O_{5\frac{1}{2}}N_4$ (12×10^{-5} moles in 1 l.).
0.0124 g. methyl pheophorbide *b*,
anhydrous form, $C_{36}H_{36}O_6N_4$ (4×10^{-5} moles in 1 l.).

The mixture is dissolved in 2 cc. of pyridine and saponified with boiling, 35 per cent, methyl alcoholic potash in exactly the same manner as described in the case of the pheophytin of the experiment above.

The fractionation is also carried out in exactly the same manner, except that the preliminary purification by transferring to 12–20 per cent acid may be omitted. There are thus obtained again 500 cc. of chlorin *e* in ether saturated, 3 per cent hydrochloric acid and 500 cc. rhodin *g* in 12 per cent hydrochloric acid. These solutions are serviceable for about a week; on standing longer than this the chlorin solution

becomes a little greener, the rhodin solution a bit yellower, which makes the colorimetric comparison somewhat difficult. In such cases it is better to repeat the comparison after the test solution has also aged a day.

For the Yellow Pigments. In spite of the similarity of their colors, carotin and xanthophyll were not allowed to replace one another in the preparation of the comparison solutions, because their color intensities are dissimilar. The color intensities, as is stated in Chapter XII, do not differ according to a fixed ratio but the ratio of the intensities varies with different depths of a particular solvent and with the same depth of different solvents.

The comparison solutions for carotin are prepared with petroleum ether; those for xanthophyll, with ether, or

> 0.0134 g. carotin in 0.5 l. of petroleum ether that contains a little alcohol (5×10^{-5} moles per l.), and
> 0.0142 g. xanthophyll in 0.5 l. of ether (5×10^{-5} moles per l.).

The carotin solution is stored in a well stoppered flask in the dark; it has been ascertained that in the course of 3 weeks it loses none of its intensity.

The xanthophyll solution, on the other hand, must be prepared fresh every day, because it fades quickly, perhaps on account of impurities in the ether. After 2 days an ethereal comparison solution had faded 5 per cent and, after 3 weeks, 60 per cent.

The preparations used were recrystallized (xanthophyll from methyl alcohol, then from chloroform; carotin from alcohol, then from petroleum ether) and their purity confirmed by elementary analysis. Both are stored in sealed tubes filled with carbon dioxide.

Substitute for the Comparison Solutions. The time consumed in the preparation of the comparison solutions, and the difficulties which are encountered by the botanist and the physiologist in producing pure preparations of the comparison substances induced us to substitute for the unstable yellow pigments easily accessible and stable pigments. This substitution offers a great advantage especially for xanthophyll, on account of the instability of its solutions.

The great similarity between the spectra of these yellow leaf pigments and that of alizarin caused us to suspect that this commercial pigment was a suitable comparison substance. But the intensity of color and the hue of alizarin depend too much upon the solvent, and

are too much influenced by traces of alkalies or any metallic impurities; thus a troublesome coating usually forms upon the walls of the glass vessels. Carotin and xanthophyll can, therefore, be compared with alizarin (a solution of 0.500 g. of alizarin in 200 cc. of chloroform, which is diluted with ether to 1 liter, is suitable) but it is not suitable as a standard substance.

A comparison with the above described standard solutions of carotin and xanthophyll shows, when the color intensities are matched in the Wolf colorimeter, for a layer of

100 mm. carotin solution = 126 mm. solution of alizarin.
100 mm. xanthophyll solution = 92 mm. solution of alizarin.

In the solvents indicated the molecular color intensity of carotin is 52 times (of xanthophyll, 38 times) stronger than that of alizarin.

An aqueous solution of potassium dichromate is a reliable comparison substance, even if its absorption spectrum does deviate more from that of an ethereal or petroleum ether solution of xanthophyll or carotin than does that of alizarin in chloroform-ether.

2 g. of potassium dichromate were dissolved in 1 l. of distilled water and the comparison solutions of carotin and xanthophyll, such as were used for the earlier determinations, were replaced in the following manner:

100 mm. carotin solution correspond to 101 mm. of the potassium dichromate solution.
50 mm. carotin solution correspond to 41 mm. of the potassium dichromate solution.
25 mm. carotin solution correspond to 19 mm. of the potassium dichromate solution.

100 mm. of xanthophyll solution correspond to 72 mm. of the potassium dichromate solution.
50 mm. of xanthophyll solution correspond to 27 mm. of the potassium dichromate solution.
25 mm. of xanthophyll solution correspond to 14 mm. of the potassium dichromate solution.

The substituted comparison solution was compared with the solutions described; that is, with those of the different preparations of carotin and xanthophyll, in a series of colorimetric determinations.

In the experiments whose results are quoted below this substitution for the real comparison solutions was not applied.

Calculation.

The colorimetric measurements are made with several readings, the cylinders are then exchanged and the readings repeated. In all determinations constant depths of the comparison solutions, which had been found suitable by test, were used; namely,

for phytochlorin 40 mm., commonly designated: h'_a
for phytorhodin 50 mm., commonly designated: h'_b
for carotin 100 mm., commonly designated: h'_c
for xanthophyll 100 mm., commonly designated: h'_x

The corresponding layers of the test solutions (0.5 l. in the case of the green, 0.1 l. for the yellow pigments) are designated h_a, h_b, h_c, h_x.

The molecular component ratio of the two chlorophylls,

$$\left(\frac{\text{chlorophyll } a}{\text{chlorophyll } b}\right)$$

is, since the comparison solutions were prepared with the ratio of 3 moles of *a*: 1 mole *b*, designated as:

$$Q_{\frac{a}{b}} = 3 \times \frac{40}{50} \times \frac{h_b}{h_a} = 2.4 \times \frac{h_b}{h_a}$$

The molecular ratio of the two yellow pigments, $\left(\frac{\text{carotin}}{\text{xanthophyll}}\right)$ is

$$Q_{\frac{c}{x}} = \frac{h_x}{h_c}.$$

Finally, the ratio of the two chlorophylls to the two yellow pigments, all expressed in moles, is of consequence:

$$Q_{\frac{a+b}{c+x}} = \frac{4\left(\frac{3h'_a}{h_a} + \frac{h'_b}{h_b}\right)}{\frac{h'_c}{h_c} + \frac{h'_x}{h_x}}.$$

Or, for the chosen depth of the comparison solutions:

$$\frac{2\left(\frac{2.4}{h_a} + \frac{1}{h_b}\right)}{\frac{1}{h_c} + \frac{1}{h_x}}.$$

The colorimetric determination gives, at the same time, the weights of the four pigments in the 20 g. of fresh leaves used; and furthermore, in 1 kg. of the same, by the following calculation:

$$\text{Chlorophyll } a = 50 \times 0.00902 \times 6 \times \frac{40}{h_a},$$

$$\text{Chlorophyll } b = 50 \times 0.00916 \times 2 \times \frac{50}{h_b},$$

$$\text{Carotin} = 50 \times 0.00536 \times \frac{1}{2} \times \frac{100}{h_c},$$

$$\text{Xanthophyll} = 50 \times 0.00568 \times \frac{1}{2} \times \frac{100}{h_x}.$$

In order to ascertain the amounts of the four pigments in one kg. of dry leaves, the per cent of dry matter in the fresh leaves was determined in almost every experiment. This we propose to designate as "dry content." The values found for the fresh leaves are consequently to be multiplied by $\frac{100}{\text{dry content}}$ in order to calculate the pigment content of the dried material.

4. Results.[12]

Twenty samples were investigated by our method for the quantitative determination of the four leaf pigments. The first results serve much more to confirm the usefulness of the analysis and to bring up questions of physiological significance that can be solved by the method than to establish possible regularities in the distribution of the pigments. The number of investigated cases is as yet much too small for this purpose. The first experiments enable us to know the ratios of the green and yellow pigments and the most important phenomena of their distribution only. The communication of our latest analyses should take precedence over the earlier observations of Willstätter and Isler, which concern the component ratio $Q_{\frac{a}{b}}$ in different plants and their chlorophyll content.

The component ratio, 2.6, which results from averaging the samples of Table I, was found to be a little too low, because of the inaccuracies disclosed in section 2b.

[12] Unpublished.

TABLE I.

COMPOSITION OF CHLOROPHYLL, AS TO ITS TWO COMPONENTS, ACCORDING TO WILLSTÄTTER AND ISLER.

Experiment	Plant	Date of collecting	10 g. of dried leaves gave		Component ratio
			Phyto-chlorin e	Phyto-rhodin g	
1	Spruce	July 21, 1911	0.0124	0.0044	2.9(1)
2	"	"	0.0124	0.0045	2.8(4)
3	Grass	June 8, 1911	0.0305	0.0140	2.2(4)
4	"	June 22, 1911	0.0313	0.0152	2.1(1)
5	Nettle	Last of May, 1911	0.0357	0.0174	2.0(9)
6	"	June 23, 1911, in the sun, 4 P. M.	0.0281	0.0104	2.7(5)
7	"	June 30, 1911	0.0228	0.0087	2.6(7)
8	"	July 20, 1911, in the sun, 2 P. M.	0.0324	0.0110	3.0(0)
9	"	July 24, 1911, in the shade, 7 A.M.	0.0409	0.0157	2.6(7)
10	"	Middle of Sept., 1911	0.0307	0.0140	2.3(5)
11	"	March 22, 1912, 7 A. M., extracted fresh	0.0333	0.0144	2.3(7)
12	"	Same as 11	0.0330	0.0131	2.5(8)
13	Plane tree	June 1, 1911, 5 P. M.	0.0316	0.0123	2.6(2)
14	" "	July 6, 1911	0.0350	0.0152	2.3(6)
15	" "	July 25, 1911, 9 A. M.	0.0504	0.0182	2.8(4)
16	Horse chestnut	June 8, 1911	0.0473	0.0184	2.6(3)
17	" "	July 4, 1911	0.0421	0.0179	2.4(1)
18	" "	July 22, 1911	0.0482	0.0167	2.9(6)
19	" "	"	0.0502	0.0171	3.0(1)
20	Hog weed	May 18, 1911	0.0322	0.0122	2.7(0)
21	" "	July 17, 1911	0.0299	0.0120	2.5(6)
22	Balm mint	July 19, 1911	0.0290	0.0118	2.5(1)
23	Elder	June 1, 1911, 5 P. M.	0.0396	0.0178	2.2(7)
24	"	July 10, 1911, 11 A. M.	0.0405	0.0188	2.2(0)

In our determinations errors were diminished to a few per cent of the ratio number found for the chlorophyll components; the parallel experiments gave, in fact, the following numbers:

No. 1 2.74 and 2.70; difference 1.4%
" 2 2.83 " 2.71; " 4.3 " (small error in saponification)
" 3 2.90 " 2.80; " 3.5 " " " " "
" 10 2.98 " 2.90; " 2.7 "
" 1a 2.07 " 2.05; " 1.0 "

TABLE II.

CHLOROPHYLL CONTENT OF DIFFERENT PLANTS ACCORDING TO WILLSTÄTTER AND ISLER.

Experiment	Plant	Date of collecting	Dry weight of 100 g. of fresh leaves	1 kg. of dry leaves contains: Components *a*	Components *b*	Chlorophyll	Chlorophyll in 1 kg. of fresh leaves
1	Spruce	July 21, 1911	50	1.87	0.67	2.54	1.27
3	Grass	June 8, 1911	28	4.62	2.10	6.71	1.88
11	Nettle	March 22, 1912	17.5	5.04	2.16	7.20	1.26
6	"	June 23, 1911	30	4.25	1.56	5.80	1.74
8	"	July 20, 1911	34	4.90	1.66	6.56	2.23
10	"	Middle Sept., 1911	.....	4.65	2.10	6.75	.....
13	Plane tree	June 1, 1911	23	4.78	1.85	6.63	1.53
15	"	July 25, 1911	24	7.63	2.73	10.37	2.49
16	Horse chestnut	June 8, 1911	29	7.16	2.77	9.93	2.83
19	"	July 22, 1911	34	7.29	2.50	10.18	3.46
20	Hog weed	May 18, 1911	20	4.87	1.83	6.70	1.34
23	Elder	June 1, 1911	21	5.99	2.67	8.66	1.82

The same is true for the ratio, $\frac{\text{carotin}}{\text{xanthophyll}}$. Its determination is even more exact than that of the green pigments.

No. 1	0.588	and	0.588;	difference	0.0%
" 2	0.629	"	0.613;	"	2.5"
" 3	0.510	"	0.513;	"	0.6"
" 9	0.653	"	0.649;	"	0.6"
" 1a	0.345	"	0.347;	"	0.6"

If, therefore, greater deviations occur between several analyses, it can now be considered as certain that they are not caused by errors of determination but that they correspond to natural variations. Upon consideration of the sources of error in our method, we maintain that it is improbable that it still involves important errors which act in one and the same direction and consequently do not appear in the differences shown by duplicate experiments. The true component ratios, therefore, can not differ very much from the values found.

The amounts of chlorophyll and carotinoids that were found by means of our colorimetric determinations are listed in Table III which follows; and the component ratios, in Table IV.

TABLE III.

THE AMOUNT OF GREEN AND YELLOW PIGMENTS IN LEAVES.

No.	Plant	Living conditions	Dry content	Amounts (in g.) in 1 kg. of fresh leaves				Amounts (in g.) in 1 kg. of dry leaves					
				Chloro-phyll *a*	Chloro-phyll *b*	Carotin	Xantho-phyll	Chloro-phyll *a*	Chloro-phyll *b*	Carotin	Xantho-phyll	Total chloro-phyll	Total yellow pigments
1	Sambucus nigra	Sun leaves	27.8	1.615	0.599	0.146	0.263	5.82	2.16	0.52	0.95	7.98	1.47
				1.616	0.607	0.145	0.262	5.82	2.18	0.52	0.94	8.00	1.46
2	" "	"	24.5	1.536	0.552	0.134	0.226	6.26	2.25	0.55	0.92	8.51	1.47
				1.514	0.559	0.133	0.230	6.18	2.28	0.54	0.94	8.46	1.48
3	" "	"	28.3	1.732	0.605	0.146	0.301	6.13	2.14	0.51	1.06	8.27	1.57
				1.725	0.628	0.144	0.298	6.10	2.22	0.51	1.05	8.32	1.56
4	Aesculus hippocastanum	"	35.5	2.517	0.885	0.291	0.444	7.09	2.49	0.82	1.25	9.58	2.07
5	" "	"	38.5	2.477	0.890	0.291	0.444	6.44	2.31	0.76	1.15	8.75	1.91
6	" "	"	37.5	2.123	0.873	0.298	0.466	5.66	2.33	0.79	1.24	7.99	2.03
7	Platanus acerifolia	"	32.0	1.692	0.489	0.106	0.233	5.29	1.53	0.33	0.73	6.82	1.06
8	" "	"	35.0	1.666	0.508	0.152	0.323	4.76	1.45	0.43	0.92	6.21	1.35
9	Fagus sylvatica	"	....	2.060	0.670	0.185	0.299	....	....	....	....	....	....
				....	....	0.186	0.302	....	....	....	....	....	....
10	Populus canadensis	"	33.5	1.395	0.476	0.097	....	4.16	1.42	0.29	....	5.58	....
				1.395	0.488	0.097	....	4.16	1.42	0.29	....	5.62	....
11	" "	"	———	1.32	0.378	0.053	....	....	....	....	....	....	....
1a	Sambucus nigra	Shade leaves	16.3	1.285	0.630	0.063	0.192	7.91	3.88	0.38	1.18	11.79	1.56
				1.304	0.645	0.063	0.192	8.02	3.97	0.39	1.18	11.99	1.57
6a	Aesculus hippocastanum	"	25.0	1.959	0.864	0.093	0.279	7.93	3.73	0.37	1.11	11.66	1.48
8a	Platanus acerifolia	"	25.0	2.112	0.679	0.127	0.311	8.45	2.70	0.51	1.25	11.15	1.76
9a	Fagus sylvatica	"	37.3	2.775	0.965	0.131	0.252	7.45	2.59	0.35	0.68	10.04	1.03

Notes on Table III.

No. 1. July 12, 1912, 5 p. m., clear weather; deep green, thick leaves without stems and midribs.

No. 2. July 15, 4 a. m. Worked up immediately after picking; saponification in the case of the first of the two determinations was somewhat less favorable (1–2 per cent too much of the weak bases).

No. 3. July 15, 5 p. m. Clear weather; leaves as in 1 and 2. There was a small saponification error in the first determination.

No. 4. July 18, 4 a. m. Leaves without midribs.

No. 5. July 18, 5 p. m. Cloudy.

No. 6. July 17, 7 a. m.; clear; fresh leaves from the top of the tree.

No. 7. July 18, 4 a. m.; the crown of the experimental tree (also that of No. 8 and 8a) was much pruned in the spring; the leaves were taken from growing sprouts, the uppermost leaves of the twigs were not used; a little too much of the weak bases was produced in the saponification (likewise in No. 8).

No. 8. July 18, 5 p. m.; cloudy; leaves as in No. 7.

No. 9. July 10, 4 p. m.; clear; leaves very rich in dry matter, difficult to grind.

No. 10. July 8, 7 a. m.; clear; leaves from the edge of the crown; leathery and difficult to work, assumed a brown color on grinding without treatment with acetone.

No. 11. July 6, 7 a. m.; clear; trunk shoots without the topmost leaves.

No. 1a. July 11, 5 p. m.; clear; leaves from the interior of the bush, very thin, partly slightly yellowish as if dying.

No. 6a. At the same time with No. 6; leaves from the very interior of the tree crown, soft and somewhat pale.

No. 8a. Like 8, but from the interior of the crown, leaves much thinner and lighter than the sun-leaves.

No. 9a. Like 9. Leaves wholly from the interior of the crown, nevertheless dark green like the sun-leaves in contrast to the shade-leaves of Sambucus and Aesculus.

Pigment Content. The chlorophyll content of the leaves of different plants ranges between 0.6 and 1.2 per cent of the dry matter and most often amounts to 0.8 per cent; that is, 0.6 per cent chlorophyll *a* and 0.2 per cent chlorophyll *b*. Much greater deviations are found with dissimilar leaves of a single plant than between the different plants. The leaves in the shade are, if the chlorophyll is referred to

TABLE IV.

COMPONENT RATIO OF THE GREEN AND THE YELLOW PIGMENTS.

No.	Plant	Living conditions	Time of day	$Q_{\frac{a}{b}}$	$Q_{\frac{c}{x}}$	$Q_{\frac{a+b}{c+x}}$
1	Sambucus nigra	Sun-leaves	5 p. m.	2.74	0.588	3.33
				2.70	0.588	3.35
2	" "	"	4 a. m.	2.83	0.629	2.83
				2.71	0.613	2.88
3	" "	"	5 p. m.	2.90	0.510	3.10
				2.80	0.513	3.04
4	Aesculus hippocastanum	"	4 a. m.	2.89	0.699	2.84
5	" "	"	5 p. m.	2.82	0.699	2.80
6	" "	"	7 a. m.	2.47	0.676	2.40
7	Platanus acerifolia	"	4 a. m.	3.52	0.478	3.98
8	" "	"	5 p. m.	3.34	0.500	2.83
9	Fagus silvatica	"	4 p. m.	3.13	0.653	3.45
				....	0.649	....
10	Populus canadensis	"	7 a. m.	2.98	....	....
				2.90	....	....
11	" "	"	7 a. m.	2.82	....	....
1a	Sambucus nigra	Shade-leaves	5 p. m.	2.07	0.345	4.63
				2.05	0.347	4.72
6a	Aesculus hippocastanum	"	7 a. m.	2.30	0.353	4.70
8a	Platanus acerifolia	"	5 p. m.	3.16	0.433	3.31
9a	Fagus silvatica	"	5 p. m.	2.92	0.553	6.02

the dry weight, much richer in chlorophyll (not also true for the carotinoids) than the sun-leaves. The comparison would result quite differently if the pigment content were referred to the leaf surface since the shade-leaves, for example in the case of Sambucus, Aesculus and Platanus, are often very thin and light.

TABLE V.

CHLOROPHYLL (IN G.) IN 1 KG. OF DRY LEAVES.

No.	Plant	Sun-leaves	Shade-leaves
1 and 1a	Sambucus nigra	7.99	11.89
6 and 6a	Aesculus hippocastanum	7.99	11.66
8 and 8a	Platanus acerifolia	6.21	11.15
9 and 9a	Fagus silvatica	$<$7.00	10.04

The content of the yellow pigments, regarding which no information has as yet been presented, also ranges within narrow limits; in the plants tested it amounts to between 0.1 and 0.2 per cent of the dry

weight, of which xanthophyll makes up 0.07–0.12 and carotin, 0.03–0.08 per cent.

The pigment content of leaves is not subject to any important fluctuation during different periods of the day; almost the same quantities of pigment are found regardless of whether the leaves are collected at the beginning or toward the close of a summer day.

TABLE VI.

PIGMENT (IN G.) IN 1 KG. OF DRY LEAVES.

No.	Plant	Chlorophyll		Carotinoids	
		4 A. M.	5 P. M.	4 A. M.	5 P. M.
2 and 3	Sambucus nigra	8.49	8.30	1.48	1.57
4 and 5	Aesculus hippocastanum	9.58	8.75	2.07	1.91
7 and 8	Platanus acerifolia	6.82	6.21	1.06	1.35

Ratio of the Chlorophyll Components. The average result of our experiments (Table IV) amounts to 2.85, with maximum differences of ± 0.7–0.8, when the individual results of the duplicate experiments are replaced by their averages. The magnitude of these deviations, which are even greater than those in the determinations of Willstätter and Isler, was dependent upon the extreme living conditions of the leaves that were used for the comparison. It appears that in the case of some plants that are poorly organized for shade growth, as, for example, Sambucus, the shade-leaves show abnormal ratios, while the beech, a true shade plant, presents in its shade-leaves no important deviation from the usual phenomenon.

If the specially selected samples of shade-leaves are omitted, the remaining component ratio determinations, for which again only the average results of the duplicate experiments are given, give an average value of 2.93, with maximum deviations of ± 0.5–0.6. This average value, therefore, gives the result of the determinations for normal living leaves.

Contrary to expectations, the time of day has no influence upon the ratio of the pigments; the ratio, therefore, does not change during assimilative activity of the chloroplasts.

The average component ratio of the shade-leaves is somewhat lower; it amounts to 2.61, with maximum deviations of ± 0.55.

TABLE VII.

	$Q_{\frac{a}{b}}$		$Q_{\frac{c}{x}}$	
	4 A.M.	5 P.M.	4 A.M.	5 P.M.
Sambucus	2.77	2.85	0.621	0.512
Aesculus	2.89	2.82	0.699	0.699
Platanus	3.52	3.34	0.478	0.500

It has thus become evident that the composition of chlorophyll from different plants, and under different growing conditions from one and the same plant, is, as regards its two components, approximately, but not exactly, constant. There are about three molecules of chlorophyll *a* to one of chlorophyll *b*.

The ratio $Q_{\frac{c}{x}}$ of the two yellow pigments, of which xanthophyll predominates, shows variations that are not much greater than those of $Q_{\frac{a}{b}}$.

The average value derived from all the determinations is 0.546, with deviations of ± 0.15–0.2. In this case, separate determinations for the leaves exposed to sunlight and for those in the shade give a greater difference than in the case of the chlorophyll components.

The former; that is, leaves under normal living conditions, show an average ratio of the yellow components of 0.603, with maximum deviations of ± 0.1; there are 1.5 to 2 molecules of xanthophyll to one molecule of carotin. Shade-leaves give an average quotient of 0.421, likewise with deviations of ± 0.1. There is a somewhat greater spread between the lowest and highest observed values than in the case of the ratio of the chlorophyll components.

Relation between the Green and Yellow Pigments. The statements regarding the amounts of the individual pigments have already indicated that the green pigments predominate over the yellow.

The ratio of chlorophyll $(a+b)$ to the yellow pigments $(c+x)$, expressed in molecules, is

As an average for all determinations 3.56
" " " of the determinations for sun leaves 3.07
" " " " " " " shade " 4.68

In the shade-leaves of several plants (Platanus is an exception) the quotient $\frac{a+b}{c+x}$ increased considerably, even to 6 in the case of a plant well organized for growth in the shade.

Of the chlorophyll components, the one poorer in oxygen is always in excess; of the carotinoids, it is always the one that contains oxygen that predominates. A simple relation has not, however, been found between $Q_{\frac{a}{b}}$ and $Q_{\frac{c}{x}}$. A mutual increase of chlorophyll *a* and xanthophyll, the reduced chlorophyll component and the oxidized carotinoid, occurs only in some of the samples.

5. Determination of the Pigments of Brown Algae.[13]

The definition of Pheophyceae pigments is much disputed. It is still uncertain whether chlorophyll, as such, is present in the brown algae and in the Diatomaceae, or whether it is present in the form of a brown colored derivative which may easily change into chlorophyll. The investigations of H. Molisch[14] have developed further to an interesting hypothesis the opinion, already presented by F. Cohn,[15] that "pheophyll," a body closely related to chlorophyll, is perhaps only a modification of chlorophyll. According to this hypothesis there is present in the Pheophyceae and in the Diatomaceae a brown chlorophyll derivative only, which changes to ordinary chlorophyll upon the sudden killing of the organism with hot water or hot air and upon the action of organic solvents. Molisch compares pheophyll with the brown pigment that is formed immediately from chlorophyll by the action of caustic potash and that so easily changes further into the green chlorophyll derivatives (chlorophyllins).

Other investigators, particularly recently M. Tswett,[16] F. Czapek[17] and H. Kylin[18] (who has published a critical investigation on the pig-

[13] From unpublished investigations of R. Willstätter and H. J. Page.

[14] Botan. Ztg. 63: 131, 1905.

[15] Some algae of Heligoland. Contributions to a Better Knowledge of Algae and their Distribution. No. 2, p. 19. Published by L. Rabenhorst. Leipzig, 1865. —Contributions to the Physiology of the Phycochromaceae and Florideae. Archiv. f. mikroskop. Anatomie, Vol. 3, 1867.

[16] Ber. d. deutsch. botan. Ges. 24: 235, 1906; also, Die Chromophylle in der Pflanzen- und Tierwelt, p. 300.

[17] Lotos. 59. 1911.

[18] Archiv. für Botanik. Stockholm. Vol. 11, No. 5, 1912, and Zeitschr. f. physiol. Chem. 82: 221, 1912.

ments of the Fucoideae), dispute the existence of pheophyll. Tswett assumes that the chlorophyll color is only masked by the yellow pigments, particularly fucoxanthin, and that it is brought to view, in the well known phenomenon of the greening of brown algae upon steeping, by the solution of the pigments in the fatty substances and, in the case of the action of solvents or reagents, by the solution or alteration of the fucoxanthin.

It is more correct to say that it is always the solution of the chlorophyll that brings about the greening of the brown algae, regardless of whether the treatment is with hot water or with solvents.

Willstätter and Page completed the proof that chlorophyll is contained even in the brown algae. In general, the brown algae show an olive green to a brownish green color; the chlorophyll color is masked in them mainly because the yellow pigments predominate quantitatively. The molecular ratio of the green to the yellow pigments is here about 1 : 1 instead of 3 to 5 : 1 as in many land plants.

If the algae contained a pigment similar to the often mentioned brown phase of chlorophyll, which is distinguished from chlorophyll probably by the opening of a lactam ring, it would not be transformed under various influences (heat, solvents) into chlorophyll itself but, according to the circumstances, into derivatives of the chlorophyllin or of the isochlorophyllin series. But this is not the case.

The view of Molisch is completely disproved by means of spectroscopical investigations. A brown pigment would certainly be quite different, optically, from chlorophyll as, in fact, the spectrum of the brown phase is, when compared with the chlorophyll spectrum. In the former no absorption is observed in the red while there is strong absorption in the green and the violet (see Chapter VI, section 4). The spectrum of the brown algae is, however, not very different from that of the common leaves. There is also, as the following measurements show, no important difference between fresh and steeped brown algae. The only differences are that, in the case of the fresh algae, the absorption bands are indistinct and the spectrum is darkened between the bands while, with steeped algae, the absorption bands are displaced somewhat toward the violet, probably because of the passage of the chlorophyll from a colloidal condition into solution in the fats and waxes. The ether extract, similarly to that prepared from ordinary leaves, shows the bands displaced still farther toward the violet.

According to Willstätter and Page the microscopical investigation of the brown algae agrees with the spectroscopical observations. Sec-

ABSORPTION SPECTRUM.

	Fucus, fresh	Fucus, steeped
Orange brown piece	685—663 . . 536—	682—665 522—
Olive colored piece	688—664 . 515 (slightly intensified about 590) 515—	683——664 shadow about 585 506—
Olive green piece	I. 695—655 . . II. 641 . . . 616 . . 610 III. 595 . . 578 IV. 561——538—	I. 687—656 . . . 653 II. 630 . 610 III. 591 . 574 IV. 545——510—

	Ethereal extract from fucus	Fucus in fresh condition
Band I	678—647	694—661 . . . 655 . .
" II	627 . . . 606	640 . . . 612 .
" III	585 . . 574	593 . . 579
" IV	545 | 525	} 555——512—
End absorption	509—	

tions of the greenest parts of fresh Fucus (figure 5) show, immediately beneath the colorless cuticle, a row of cells a large part of whose pigment is found in little individual grains in the half of the cell that is farthest from the cuticle; that is, differently than in green leaves. The half of the cell that is farthest from the cuticle is olive green; the other half is strongly refractive and, most often, pale yellow. After treatment with hot water the pigment has diffused and is uniformly distributed throughout the whole cell; with the exception of a colorless

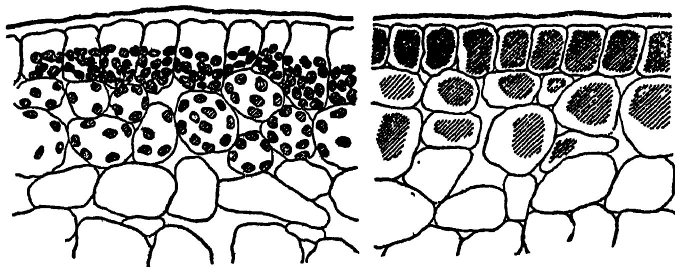


FIG. 5. Microscopical sections of Fucus. On the left in a fresh condition; on the right, steeped.

border it is clearer and more distinctly green. The solution of the pigment in the oily content of the pigment-containing cells is, therefore, simultaneous with the change of color and spectrum on steeping.

Sections of orange brown pieces of Fucus give another picture. Here, the outermost layer of cells is rather completely filled with pigment and, in this case, the outer half of the cell is deep brown and the inner, frequently, lighter brown. Beneath this first layer the picture is less regular; there are cells with individual, but not numerous, olive green chlorophyll bodies. Such pieces of algae become only a little more green and somewhat lighter in color on steeping in boiling water; the pigment is then distributed uniformly in the first layer of cells.

Intermediate between the olive green and red brown sections as described are the sections of olive brown pieces. They show yellow pigment, apparently dissolved, diffused throughout the entire cells in the outer layer, while the darker inner half of the cell contains more of the pigments, and, in fact, all the chlorophyll, in the granules.

A second question is that of the composition of the chlorophyll extracted from the Pheophyceae.

H. C. Sorby,[19] as well as M. Tswett,[20] have assumed that there is present one of the two components of ordinary chlorophyll; namely *a*, together with a third chlorophyll component, which is called chlorofucin by Sorby and chlorophyll γ by Tswett. We are able to trace this view back to the preliminary note of G. G. Stokes[21] (1864) wherein he spoke of two chlorophyll components and continued as follows:

" . . . but in olive-colored seaweeds (Melanospermae) the second green substance is replaced by a third green substance, and the first yellow substance by a third yellow substance, to the presence of which the dull color of these plants is due."

Tswett gives for the assumed third chlorophyll component a very characteristic spectrum,

I 638—622, II 588—575, III 465—440 μμ (in ether),

which shows a sharp band exactly in the interval between the absorption bands of chlorophyll *a* and *b* in the orange. According to Tswett the same band is to be found in the spectrum of fresh brown algae at about 640–625 μμ, although, in accordance with the general displace-

19 Proc. Roy. Soc. 21: 442, 1873.

20 Ber. d. deutsch. botan. Ges. 24: 235, 1906.

21 Proc. Roy. Soc. 13: 144, 1864.

ment of the absorption bands, it ought to have been displaced in the leaves considerably farther toward the red end.

Willstätter and Page obtained no trace of this third component of chlorophyll when working up quickly fresh brown algae with cold solvents; although they succeeded in bringing the entire pigment into solution, their extracts did not show an absorption band at about 630. Only from no longer fresh, or dried, Pheophyceae were solutions repeatedly obtained that showed this chlorophyll derivative which is formed by a still unknown reaction. On storage of the brown algae the chlorophyll deteriorated more and more; the pigments in the meal of dried brown algae were also but slightly stable.

The assumed third component is not a natural pigment.

In spite of the absence of a special component the chlorophyll of the Pheophyceae shows a very remarkable deviation from that of land plants, as well as from that of the green algae. It consists almost exclusively of the *a* component. Only traces of chlorophyll *b* are to be observed; at most 5 per cent.

The chlorophyll component *a*, isolated from the Fucoideae by Willstätter and Page, agrees, in its magnesium and phytol content and in the composition of the nitrogen-containing cleavage products, with the pigment from other plants.

With regard to the yellow pigments of the Pheophyceae the original statements of Stokes have already been confirmed by Tswett and by Kylin.

The brown algae contain three nitrogen-free pigments; carotin, xanthophyll (this, according to Tswett, is somewhat different from ordinary xanthophyll and is designated by him fucoxanthophyll) and fucoxanthin (also called phycoxanthin). The isolation and the analysis of fucoxanthin is set forth in Chapter XII, section 4; it is richer in oxygen than the other carotinoids; its formula is $C_{40}H_{56}O_6$. This pigment, peculiar to the brown algae, was probably first observed by Stokes, then by A. Millardet[22] and by Sorby, but was not isolated; other investigators (Hansen, Gaidukov) have disputed its existence. Tswett and Kylin first succeeded in separating fucoxanthin in solution from xanthophyll and in describing the properties of the solution.

A brown pigment, phycophein, assumed by many botanists, does not exist in the living brown algae; Molisch, Tswett and Kylin have clearly shown this. Upon quick extraction with aqueous acetone so

22 Compt. rend. 68: 462, 1869.

that oxidase action is excluded, no water soluble pigment is obtained and the extracted plant matter is practically colorless.

The determination of all the pigments of the Pheophyceae according to the method of Willstätter and Page depends upon our general method of analysis.

Xanthophyll and fucoxanthin can be separated practically quantitatively by a somewhat complicated process of distribution between ether-petroleum ether and 70 per cent methyl alcohol. By repeated fractional separation the solution of fucoxanthin becomes homogeneous since this passes over into the aqueous alcohol much more easily than does xanthophyll. The mixture of chlorophyll and yellow pigments that remains is fractionated as usual.

Method of Analysis. The brown algae are pressed out between filter paper and finely ground in a syenite mill. A portion of the comminuted algae is used for the determination of the dry weight and 40 grams for extraction. For this the material is ground with about 200 grams of sand (even more for especially tough algae) and 50 cc. of 40 per cent acetone, then with 50 cc. more of 30 per cent acetone. After transfer to a "Nutsch" and preliminary extraction with more 30 per cent acetone, the total pigment is extracted with anhydrous acetone; the filtrate is at first yellow, then yellowish green, blue green and finally colorless. The pigment is transferred from the extract into an ethereal solution by admixture with 300 cc. of ether and dilution with distilled water. The ethereal solution is freed from acetone by very careful washing with distilled water. It is then mixed with an equal volume of low boiling petroleum ether.

For the separation of the fucoxanthin the ether-petroleum ether solution is shaken four times with an equal volume of 70 per cent methyl alcohol that has been saturated with petroleum ether; the volume of the upper layer is kept constant by the addition of ether. Since xanthophyll has also passed over into the aqueous methyl alcohol the united extracts are washed once with an equal volume of a mixture of five volumes of petroleum ether and one volume of ether. A little fucoxanthin, however, is lost in this process and this extract is therefore concentrated in a vacuum to 250 cc.; an equal volume of ether is added and two more extractions are made, each with 500 cc. of 70 per cent methyl alcohol that contains petroleum ether. The new methyl alcoholic extracts are added to those previously obtained and the ether-petroleum ether solution that remains is added to the main solution.

CONTENT OF THE BROWN ALGAE IN CHLOROPHYLL AND CAROTINOIDS.

No.	Genus	Time	Dry content	Quantities (g.) in 1 kg. of fresh algae				Quantities (g.) in 1 kg. of dry algae				
				Chlorophyll	Fucoxanthin	Carotin	Xanthophyll	Chlorophyll	Fucoxanthin	Carotin	Xanthophyll	Total yellow pigments
1	Fucus	May	28.5	0.503[23]	0.169	0.089	0.087	1.765	0.593	0.312	0.305	1.210
2	Dictyota	"	......	0.640	0.250	0.057	0.063	....	....	....	....	....
3	Laminaria[24]	June	15.4	0.185	0.081	0.006	0.038	1.202	0.528	0.038	0.247	0.813

The fucoxanthin is finally transferred to 250 cc. of ether for its determination, washed free from methyl alcohol and analyzed colorimetrically by means of a comparison solution of pure fucoxanthin in ether.

Half of the petroleum ether-ether solution serves for the determination of chlorophyll, the other half for the separation of carotin and xanthophyll.

Results. The Pheophyceae contain much more yellow pigments than do land plants and green algae; the molecular ratio of chlorophyll to the carotinoids is not far from one. Among the yellow pigments, fucoxanthin, which is the richest in oxygen, predominates.

MOLECULAR RATIO BETWEEN CHLOROPHYLL AND THE YELLOW PIGMENTS.

No.	Genus of algae	$Q\frac{\text{Chlorophyll}}{\text{Carotin} + \text{Xanthophyll} + \text{Fucoxanthin}}$	Carotin: Xanthophyll: Fucoxanthin
1	Fucus	0.95	1.08: 1: 1.75
2	Dictyota	1.20	0.77: 1: 3.60
3	Laminaria	1.07	0.16: 1: 1.92

The conclusion from spectroscopical observations and preparative work that the green pigment in brown algae is almost entirely chlorophyll *a* was confirmed by the quantitative determinations. After the saponification of the pheophytin and the isolation of the phytochlorin *e* during the process of analysis, the ethereal solution, which was now always only very slightly colored, was extracted with 12 per cent

23 Chlorophyll *a*; the total chlorophyll was about 5 per cent more.

24 The average values from two determinations.

hydrochloric acid and this extract was compared with the small quantity of feebly basic cleavage product that appears in the preparation of the comparison solution (in this case from methyl pheophorbide *a* alone). The fraction in the 12 per cent hydrochloric acid was, in the test, but very little stronger than in the comparison solution, although it had to contain, in the test, all the rhodin that was present in addition to some feebly basic phytochlorin. It contained merely a trace of phytorhodin *g*.

Ulva lactuca was investigated, as an example from the Chlorophyceae group, for comparison with the brown algae. The ratio of the chlorophyll components in this seaweed deviates, though not far from the average value (about 3), towards a smaller value, just as in the case of the Pheophyceae. That is, the chlorophyll is relatively rich in the *b* component. The yellow pigments are present in relatively greater proportions than in the higher plants, although not to the same extent as in the brown algae.

Ulva lactuca (Rovigno, May). Dry content, 17.6 per cent.

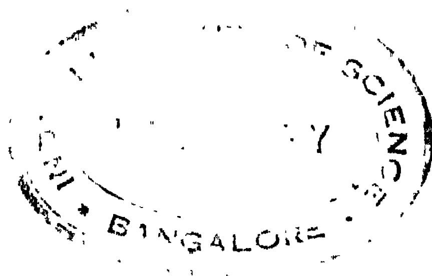
Quantities in 1 kg. of fresh algae:

0.1648 g. chlorophyll *a*, 0.1172 g. chlorophyll *b*, 0.0243 g. carotin, 0.0643 g. xanthophyll.

Quantities in 1 kg. of dry algae:

0.9362 g. chlorophyll *a*, 0.6660 g. chlorophyll *b*, 0.1381 g. carotin, 0.3653 g. xanthophyll.

$$Q_{\frac{a}{b}} = 1.43; \quad Q_{\frac{c}{x}} = 0.40; \quad Q_{\frac{a+b}{c+x}} = 1.96.$$


BANGALORE

V. PREPARATION OF CHLOROPHYLL.

1. Method of Willstätter and Hug.[1]

The Principles of the Isolation.

In order to isolate chlorophyll in an undamaged condition and free from accompanying materials it was first necessary to become acquainted with its characteristics. The requirements which the pigment must satisfy in the individual phases of the isolation and in the isolated condition were thus determined. In the first place the yellow pigments of the chloroplasts are mixed with chlorophyll; as long as they accompany the chlorophyll yellow colored solutions are obtained upon saponification with alcoholic potash and extraction with ether. Colorless materials, fats, waxes and salts of fatty acids accompany the chlorophyll and distribute themselves among different solvents in approximately the same ratio as chlorophyll; they reduce the magnesium content of the pigment and add an admixture of lime to the ash. The solubility ratios offered no means for the separation of chlorophyll from its accompanying materials. The pigment appeared to be easily soluble in all organic solvents.

The process of isolation is so conducted that the effect of each treatment upon the chlorophyll is tested by the colorimetric determination of the degree of its purity, for which crystallized ethyl chlorophyllide forms the basis. But this single test is not sufficient. In many preliminary experiments chlorophyll preparations were obtained by us which according to colorimetric analysis were almost 100 per cent pure and of which approximately one-third consisted of phytol but which nevertheless did not consist of unaltered chlorophyll. These preparations were distinguished from the chlorophyll of good leaf extracts by two properties, both of which chlorophyll very easily loses when its solutions stand, or on evaporation, on absorption by adsorbing media and, in general, in all operations. There is no betrayal of these changes by any striking difference in the spectrum. These changes were con-

[1] Paper XV.

sequently not observed by other authors who endeavored to isolate chlorophyll.

As long as chlorophyll is intact it shows the striking color change to brown upon saponification with alcoholic potash; this change of color lasts a few minutes till the saponification is complete and then the chlorophyll color returns. The brown phase is lacking in solutions and preparations if the chlorophyll has undergone allomerization as a result of a change in its lactam group. This alteration is evident in a further variation. Cleavage no longer produces the normal mixture of phytochlorin *e* and phytorhodin *g* but weaker bases.

In good alcoholic extracts of dried leaves the chlorophyll is diluted with accompanying materials about six fold. The concentration of the pigment is doubled if it is transferred from the extract into petroleum ether. After a preliminary treatment of the leaves with solvents that extract the accompanying materials but no chlorophyll (benzol is best), extracts are obtained which, although they are not of a higher degree of purity, nevertheless give a much more favorable result on fractional separation by petroleum ether. These extracts contain chlorophyll that has even less than 1.5 times its weight of accompanying material. The degree of purity can be increased to 60 by washing the crude petroleum ether solution with methyl alcohol and to even more than 60 by extracting the pigment from the petroleum ether with the same solvent that has served for washing and again transferring into petroleum ether. In this manner we obtain solutions of 70 per cent chlorophyll which can not be improved by further treatment with solvents.

If chlorophyll has reached this degree of purity its solubility is no longer influenced by admixtures as at first. Surprisingly, it is now soluble in petroleum ether only as long as this contains ethyl or methyl alcohol. If the alcohol is washed out quantitatively the petroleum ether solution becomes turbid and opalescent and the liquid then contains the chlorophyll in an extremely finely precipitated state. The chlorophyll solution appears to be ruined, but upon the addition of alcohol or ether a splendid green solution is again obtained.

Simultaneously with Willstätter and Hug[2] the Russian botanist, M. Tswett,[3] in his publication "Chromophylls in the Plant and Animal Kingdom" (Russian), made the important observation that chlorophyll, after purification of its petroleum ether solution, is precipitated

[2] Ann. d. Chem. 378: 21, 1910.

[3] Warsaw 1910; pg. 206.

therefrom by removal of the alcohol and is then no longer soluble in ligroin.

This separation from the purified solution is the basis of the method of isolation.

Once precipitated, the preparation may be further improved by reprecipitation from alcohol by a salt solution and by repeated reprecipitations from concentrated ethereal solutions by means of petroleum ether.

The isolated chlorophyll, in quickly conducted successful experiments, is pure and according to tests for all the known characteristics is unchanged.

The yield was only very small on account of the great loss of chlorophyll in the process of fractionation from its accompanying materials; it was about 5–7 per cent of the chlorophyll content of the leaves.

The Method of Procedure.

After preliminary extraction of the leaf meal with benzol and, in order to remove this, by petroleum ether, extracts were prepared by the "Nutsch" method with 96 per cent alcohol (see Chapter III, section 2a). The chlorophyll was transferred into petroleum ether, whereby extracts of 14–17 per cent purity produced crude solutions of 30–44 per cent purity. On washing with aqueous alcohol the chlorophyll concentration increased in the petroleum ether layer to a 50–60 per cent purity but no more than this.

Although the methods of separation used by Stokes, Sorby and Kraus always served for the separation of the different pigments; that is, for those separations that are observable by the eye, the process of separation in this case has an essentially new action in bringing about the separation of colorless admixtures; that is, in increasing the degree of purity.

Methyl alcohol is preferable to ethyl alcohol for the fractionation of crude chlorophyll solutions. Chlorophyll is more difficultly soluble in methyl alcohol. Furthermore, as the following experiment shows, petroleum ether is much less soluble in aqueous methyl alcohol than in aqueous ethyl alcohol and hence fractionation is easier and sharper with methyl than with ethyl alcohol.

Each 50 cc. of absolute alcohol and commercial methyl alcohol were mixed with 50 cc. of petroleum ether (B. P. 30–50°). Upon the addition of water, petroleum ether separates in the following manner:

Water added	Petroleum ether from the mixture with CH_3OH	Petroleum ether from the mixture with C_2H_5OH
0.7 cc.	10 cc.	0 cc.
1.0 "	20 "	0 "
3.0 "	40 "	0 "
5.0 "	45 "	22 "
10.0 "	50 "	43 "

Before using the aqueous methyl alcohol it is saturated with petroleum ether (B. P. 30–50°).

Chlorophyll and its accompanying materials were consequently distributed between 90 per cent methyl alcohol and petroleum ether by several consecutive fractionations, for the petroleum ether solution which was once fractionated could still be considerably improved by repeated washing.

For example, a washed petroleum ether solution, which contained 4.8 g. of 52 per cent chlorophyll in 500 cc., on a second washing with half its volume of 90 per cent methyl alcohol gave up 10 per cent of its chlorophyll to the latter with a degree of purity of 19. By this means the concentration of the chlorophyll in the petroleum ether was increased to 60.

Continued washing, however, led to a point where a higher per cent chlorophyll than that remaining in the petroleum ether began to pass over into the 95 per cent methyl alcohol.

By way of example, a purified petroleum ether solution with 8 g. of 55 per cent chlorophyll in 2 l. was employed and the distribution of the chlorophyll upon thorough shaking with an equal volume of 95 per cent methyl alcohol saturated with petroleum ether was investigated.

	Chlorophyll	Residue	Degree of purity or per cent purity
1st experiment:			
Petroleum ether solution used	8.0 g.	.....	55
Fraction in 95% methyl alcohol	3.7 g.	5.8 g.	64
Petroleum ether residue	4.5 g.	9.2 g.	49
2nd experiment:			
Petroleum ether solution used	8.5	15.3	55
Fraction in 95% methyl alcohol	4.5	6.5	69
Petroleum ether residue	4.0	8.7	46

This result was utilized to finally obtain the chlorophyll (by transferring it to 95 per cent methyl alcohol and again extracting it from

this with petroleum ether and the addition of much water) in such a pure state that it precipitated when the methyl alcohol was completely washed out.

Example.

2 kg. of stinging nettle meal, rich in chlorophyll (chlorophyll content 7 to 8 g. per kilogram) are spread, with suction but without moistening, upon a "Nutsch" and extracted with 7 l. of benzol; this requires 2–3 hours when a vacuum pump is used; the benzol that is still retained by the powder is then removed in approximately an hour's time by means of petroleum ether, for which purpose 2–2.5 liters are sufficient. After filtering under strong suction, the chlorophyll is extracted from the meal upon the suction filter with 3 l. of 96 per cent alcohol in the course of about 2 hours. The extract, which contains much petroleum ether, amounts to about 3 liters. It is at once worked up by being mixed with 1/2–2/3 of its volume of petroleum ether (sp. gr. 0.64–0.66) and then with 1/3 of its volume of water. In this separation, the greater portion of the chlorophyll passes into the upper layer with a beautiful bluish green color. The aqueous alcoholic fraction is discolored and is light yellowish green or decidedly yellow; it is thrown away.

The crude petroleum ether solution is purified by shaking it three times, each time with half its volume of 90 per cent methyl alcohol that has been saturated with petroleum ether. The first extract is still yellowish in tinge and gives a yellow stain; the third is purer in color. In this purifying operation the loss of chlorophyll is often considerable.

The pigment is transferred from the solution, which has been washed three times, to methyl alcohol by two extractions, each with an equal volume (1–1.5 l.) of 95 per cent methyl alcohol that has been previously saturated with petroleum ether. A little less than half of the chlorophyll remains in the petroleum ether, giving it a bright blue green color; this residual solution is of value for obtaining chlorophyll derivatives, but only the purer methyl alcoholic fraction should be used for the isolation of chlorophyll itself.

The chlorophyll is again quantitatively brought into 1 l. of petroleum ether by means of a dilute solution of common salt. After washing once with 500 cc. of 90 per cent methyl alcohol this solution shows a purity of about 70 and a chlorophyll content of 2–3 grams. The beautiful bluish green solution is washed with considerable water until

the green color and the red fluorescence have disappeared and the solution has become opalescent and turbid.

The extremely finely divided, precipitated pigment is collected with considerable sodium sulphate and is subsequently washed with very volatile petroleum ether. The work should be completed at least to this point in one day.

The purity of the chlorophyll is increased by reprecipitating it from alcohol with a salt solution and further, from ether, by means of petroleum ether in the manner that has been carefully described in the original paper. The yield amounts to 0.75 to 1.0 g. but two charges may be run simultaneously at first and united for the last operations. All preparations do not produce faultless products; it is absolutely necessary to test each one individually.

2. Pure Chlorophyll According to the Method of Willstätter and Stoll.[4]

The isolation of chlorophyll by the method of Willstätter and Hug is carried out with difficulty and produces a small yield of chlorophyll with a very great expenditure of solvents and time. The purity of the preparation, endangered by the long sequence of procedures in alcoholic solutions, does not always prove to be uniformly satisfactory.

By applying new methods of extraction with aqueous acetone and a thin layer of leaf meal the method was essentially changed and so improved that it had only one point in common with the method described by Willstätter and Hug: the precipitation of the chlorophyll from the purified petroleum ether solution.

The aqueous acetone extract contains at the very beginning a better yield of chlorophyll and is, therefore, a more suitable starting material; besides, the chlorophyll is transferred to petroleum ether more easily and more completely from it than from alcoholic extracts. In particular, the nature of the admixtures that are contained in the crude petroleum ether solutions produced is quite different from that of those that are contained in the extracts made with anhydrous solvents or with solvents that contain less water. The aqueous extracts, being poorer solvents of fats and waxes, do not carry along with them the disturbing accompanying materials which increase the solubility of chlorophyll in petroleum ether and which make its precipitation so difficult that it is only possible by means of a complicated fractionation.

[4] Unpublished.

We can now precipitate the pigment in a still impure condition by simply transferring it from the extract into petroleum ether and washing away the acetone. No application is made of this since it is possible to obtain pure preparations with but little more work. Purification of the petroleum ether solution twice with 80 per cent acetone and repeated fractional separation with 80 per cent methyl alcohol, whereby xanthophyll is removed, are sufficient to increase the degree of purity just as much as was done formerly by a great number of fractionations with stronger methyl alcohol that removed more of the chlorophyll, and just as well as by transferring the chlorophyll into methyl alcohol.

The following numbers show the increase of the degree of purity with a small loss of pigment:

a. Acetone extract from 2 kg.; b. purified petroleum ether solution.

a. 3.7 l.; chlorophyll content, 16.8 g.; dry residue, 135 g.; degree of purity, 12.

b. 3.0 l.; chlorophyll content, 14.3 g.; dry residue, 21.9 g.; degree of purity, 65.

Since the isolation takes place without fractionation and decomposition (*cf.* the "residual solution" in section 1) of the material, the chlorophyll retains the natural ratio of the mixed components and the yield becomes nearly quantitative; that is, as much is obtained from 1 kg. of leaves as was formerly obtained from 13 kg.

The chlorophyll also remains unchanged as to its sensitive phytochromin groups since it comes in contact with alcohols to a slight extent only.

Method of Procedure.

A half day is required for the elaboration of 2 kg. of stinging nettle up to the point of the reprecipitation of the isolated preparation. The medium fine meal of carefully collected and dried leaves is used.

2 kg. are sucked fast upon the stoneware "Nutsch" with a pump and extracted in a half hour with 6–6.4 l. of 80 per cent (by volume) acetone. Two l. of the solvent are first allowed, without suction, to sink in for about 5 min. and then the remainder of the acetone is added, liter by liter, alternately macerating without the use of the vacuum and draining with but moderate suction. The decolorized meal is finally sucked dry by the strong action of the pump.

The pigment is transferred from the beautiful extract into 4 l. of petroleum ether (Kahlbaum's 0.64–0.66) by pouring half of the solution at a time into the whole quantity of petroleum ether in a 7 l. separatory funnel and, while rotating, slowly adding about a half liter of water each time. After drawing off the slightly yellowish green, lower layer, the petroleum ether solution is fractionally separated twice, each time with 1 l. of 80 per cent acetone; this removes impurities but very little chlorophyll. The petroleum ether layer, by taking up acetone, has increased to 6 l. The acetone is carefully removed from it by extracting 4 times, each time with a half liter of water, using gentle rotation. The first of these fractional separations removes 0.6, the second 0.5, the third 0.4 and the last 0.2 l. of acetone. By this method of fractional separation accompanying materials are removed along with the high per cent of acetone.

It is not our intention to wash out the acetone quantitatively at this point for all the chlorophyll and the xanthophyll would be precipitated and purification would be more difficult. It is convenient to separate the xanthophyll first, which is done without too great loss of chlorophyll by extracting with 80 per cent methyl alcohol. Extract three times, each time with 2 l. of 80 per cent methyl alcohol, and if the last extract still contains considerable yellow material extract a fourth and a fifth time. These extracts are easily worked up for xanthophyll; they produce 0.8 g. of it.

The last traces of methyl alcohol and acetone are removed from the petroleum ether, whose volume finally amounts to 3.6 l., by washing with water about 4 times, using 2 l. each time. The petroleum ether solution loses its fluorescence during this process, becomes turbid, and the chlorophyll precipitates. The suspension in petroleum ether is shaken with some anhydrous sodium sulfate and about 150 g. of talc; it is filtered, using a pump, through a layer of 50 g. of talc. In this process the fine precipitate easily forms a coherent layer over the talc which hinders the filtration; it is stirred up from time to time with a silver spatula.

The filtered petroleum ether solution is pale olive green to yellowish green and contains, in addition to a little chlorophyll and some oily materials, much carotin which can be easily isolated from it.

The chlorophyll-containing talc is first washed upon the "Nutsch" with ordinary petroleum ether till the latter runs off with only a pale yellow color and then with 300 cc. of a petroleum ether fraction, which

boils at 30–50°, for the removal of the difficultly volatile constituents. The petroleum ether is thoroughly removed by suction and the chlorophyll is immediately dissolved from the talc upon the suction filter by means of 1 l. of carefully distilled ether. The ethereal solution of chlorophyll is filtered through anhydrous sodium sulfate, concentrated to 100 cc., filtered once more for safety and evaporated to 25 cc. The chlorophyll is then precipitated by the slow addition of 0.8 l. of low-boiling petroleum ether. The precipitate most often forms a filterable, blue black powder but occasionally a suspension of such fine particles is formed that it can be filtered well only upon talc. It is then extracted again with ether and the solution, concentrated to 20 cc., is dried in a dish within a desiccator to the form of thin, shiny, steel blue crusts.

The mother liquor from the reprecipitation no longer has much admixed matter to be separated; for example, it contains 0.15 g. of chlorophyll in 0.5 g. of the dry residue.

In repeated experiments the yield amounted to 13 g., or 6.5 g. from 1 kg. of leaves; that is, three-fourths of their chlorophyll content. From quantitative determinations made in the form of phytochlorin and phytorhodin, the component ratio was found to be 2.4 and the degree of purity 98; in other preparations with smaller quantities a still higher degree of purity was obtained.

3. Crude Chlorophyll.[5]

The pronounced superiority of the extractions with aqueous acetone, as contrasted with the older methods, is also shown on the separation of crude chlorophyll by dilution. Willstätter and Fritzsche had already used the precipitate from alcoholic extracts as a starting material for preparing phyllophyllin but such products contained the pigment diluted by much accompanying materials. Extracts obtained by means of 80 per cent acetone (by volume) behave entirely differently. Even with a very small addition of water the pigment precipitates completely with a degree of purity of about 50. It is pure enough to be precipitated from petroleum ether, since it is just those accompanying materials that are ordinarily responsible for the miscibility of the impure chlorophyll with petroleum ether that are lacking in the aqueous acetone. A single reprecipitation from ether by means of petro-

[5] Unpublished.

leum ether is, therefore, sufficient to obtain, with only a small loss, crude chlorophyll with a degree of purity of about 90.

This is, therefore, a very simple and rapid method by means of which chlorophyll is made quite easily available for technical uses and for many preparative purposes; for example, for the preparation of pure chlorophyll and its components as well as of the phyllins and the porphyrins. The recovery of the solvent is much easier here than in the case of the isolation of pure chlorophyll since no mixtures of solvents are formed.

The increase in the concentration of chlorophyll in the four phases of the experiment is shown by the following numbers:

1 kg. of stinging nettles contains 7.5 g. of chlorophyll; concentration of the same, 0.75.

1 l. of extract contains 7.2 g. of chlorophyll; degree of purity, 7.5.

14 g. of the first precipitate contain 7.0 g. of chlorophyll; degree of purity, 50.

6.2 g. of crude chlorophyll contain 5.9 g. of chlorophyll; degree of purity, 95.

In the manner already described we extracted in 30–45 minutes upon a stoneware "Nutsch" (50 cm. diameter), with 6 liters of 78 per cent acetone, 2 kg. of stinging nettle meal which we had collected ourselves. The meal that remains is straw yellow in color and, at most, only slightly green in the lower layers.

The extract (4 l.) is supersaturated with chlorophyll, of which it contains 16–17 g.; a part of the pigments often separates in a viscous form on standing. Often, for example, in working up rather large charges of technical meal, it is advantageous to dilute the extract with 80 per cent acetone (using about 1/4 to 1/2 of its volume). The extract is shaken with 300–400 g. of talc (not too finely ground), which immediately absorbs considerable pigment. The separation of the pigment is completed by the gradual addition of 1.2 l. of water; the liquor then contains 40 per cent (by volume) of water and remains only yellowish green in color. It is immediately filtered through a thin layer of talc so that accompanying materials that precipitate slowly are not removed with the pigment.

The dark grayish green talc is washed with 2–3 l. of 65 per cent acetone, whereby rather large amounts of yellow pigment are separated and but little green pigment is lost, and then several times with

water (in all about 4 l.), the talc being stirred. A sample of the filtrate must no longer smell of acetone since this, even in traces, would interfere with the reprecipitation. The talc is now filtered as dry as possible by suction and the pigment that it contains is brought into solution by shaking in a powder flask with ether to which anhydrous sodium sulphate has been added. The solution is removed by filtering under suction; it is again filtered through sodium sulphate and evaporated to a sirupy consistency. The mass is then mixed with a little petroleum ether and the greater portion is gradually precipitated by shaking with much petroleum ether, in all 1.5–2 l. The suspension settles on standing. It is easily filterable and is washed with very volatile petroleum ether.

The yield amounts to 12–14 g. and the degree of purity usually lies between 90 and 95.

The preparation does not contain any carotin but there are present a few per cent of xanthophyll. This is obtained in the preparation of pure chlorophyll or of chlorophyllin salts from the crude chlorophyll (for example, 1 g. from 40 g.).

The petroleum ether mother liquor from the reprecipitation still contains 2–3 g. of chlorophyll; this may be separated by washing twice with 80 per cent methyl alcohol, then with water, and the isolation of all the chlorophyll may be thus completed.

The crude chlorophyll, apart from its colorless and yellow accompanying materials, answers the purity tests (phase and cleavage) just as well as do the preparations described in sections 1, 2 and 4.

4. Chlorophyll from Fresh Leaves.[6]

The isolation of chlorophyll from fresh leaves is described in order to show that the pigment that is obtained from the dried leaves is present in its unchanged natural state and in order to find out whether the pigment undergoes any changes as a result of certain influences upon the leaf cells; for example, steeping or storage of the freshly picked leaves. The procedure for working up fresh leaves is required for the investigation of the pigment from many plants that can not be easily dried without undergoing alterations.

Extracts with aqueous acetone from fresh leaves are much more dilute than those from dried leaves because the solvent action of the

[6] Unpublished.

accompanying material is not fully utilized. The preliminary extraction that is necessary for dehydration carries away much of the accompanying materials.

1 kg. of dried stinging nettle leaves gives 2 l. of extract, containing over 100 g. of dry residue.
5 kg. of fresh stinging nettle leaves gives 8 l. of extract containing 45 g. of dry residue.

The accompanying materials that passed, together with the chlorophyll, from the concentrated, aqueous acetone extract of the dried leaves into the petroleum ether did not, because of their nature, interfere with the separation of the pigment. In working up dilute extracts of fresh leaves the quantity of accompanying materials that is contained in the petroleum ether, even after washing with acetone and methyl alcohol, is not small, but even these do not have an unfavorable influence upon the separation of the chlorophyll. Extraction by shaking with acetone and methyl alcohol is sufficient to bring the degree of purity to 50, which permits precipitation of the chlorophyll from the petroleum ether.

An acetone extract from fresh stinging nettle leaves contains 4.7 g. of chlorophyll, 22.6 g. dry residue.
The petroleum ether, after washing with acetone, contains 4.5 g. of chlorophyll, 11.6 g. dry residue.
After washing with methyl alcohol it contains 4.2 g. of chlorophyll, 9.3 g. of dry residue.
The petroleum ether after precipitation contains 0.0 g. of chlorophyll, 4.4 g. dry residue.

Example. 2.5 kg. of fresh stinging nettle leaves were quickly (in 20 min.) worked up in a stone roller mill to a thin mash and this was dehydrated by shaking in a flask with 1.5 l. of anhydrous acetone and subjected to a preliminary extraction (see Chapter III, section 2c). With the use of suction and the application of strong pressure 2.6 l. of preliminary extract, containing 90 g. of dry substance, ran off. The reground press cake was now extracted with 1.2 l. of pure acetone, which was diluted to about 80 per cent (by volume) by the water content of the cake, and the extraction was continued by the addition of another liter of 80 per cent acetone. Filtering off under suction and subsequent washing with 2 l. of the same solvent (80 per cent acetone)

gave 3.8 l. of extract which contained almost all the chlorophyll content of the leaves while a considerable portion of the yellow pigments remained in the meal; by a subsequent extraction of the leaves with petroleum ether 0.06 g. of pure carotin was isolated and the petroleum ether mother liquor from the chlorophyll furnished 0.02 g. more.

The extract was permitted to flow into 1.5 l. of petroleum ether during rotation of this, whereupon the layers became sharply separated, the one beneath remaining very slightly colored. The petroleum ether solution was washed once with 0.5 l. of 80 per cent acetone and the layer of chlorophyll solution, which had increased to 3.1 l., was washed free from the greater portion of the acetone by two washings, each with 0.5 l. of water. The volume now amounted to 1.7 l. Two washings, each with 0.5 l. of 80 per cent methyl alcohol, now served chiefly for the removal of xanthophyll. There was isolated from this methyl alcoholic layer 0.15 g. of pure xanthophyll.

The loss of chlorophyll in all the fractional separations was small; at the end the solution still contained 4.2 g. of chlorophyll and this was quantitatively separated by washing five times, each time with 2 l. of water. The flocculent suspension was collected by means of 50 g. of talc, filtered upon talc, and freed from mother liquor by washing with petroleum ether. Extraction of the chlorophyll from the talc by means of ether and its slow precipitation from the concentrated solution with petroleum ether gave a yield of 4.05 g.; that is, fully four-fifths of the chlorophyll present in the leaves; a quantitative determination of the component ratio gave 2.8 and the degree of purity 97.

The pigment was obtained in the same manner from 5 kg. of fresh leaves that had been steeped for 5 min. in 20 l. of boiling, distilled water and from 2.5 kg. of stinging nettle leaves that had been stored for 4 days in an ice chest. No deviations were found to occur either during the process of isolation or in the properties of the preparation.

The work as described required a half day; if such a high yield is not required, the pigment can be isolated much more quickly; namely, in 45 minutes, from smaller quantities of fresh leaves by shortening all the operations.

The chlorophyll that is thus obtained agrees well with the other preparations; it is characterized by the purity of its solution and of its cleavage products.

Example. 250 g. of fresh nettle leaves are ground in 3–4 minutes in a syenite mill, each handful passing through the rolls twice, and

then immediately dropped into 90 per cent acetone. In order to save time the preliminary extraction is omitted and the mass is extracted in a flask for two minutes with one liter of the solvent.

After filtering under suction and subsequent washing with 0.25 l. of 80 per cent acetone, the filtrate is allowed to run into 300 cc. of petroleum ether and the chlorophyll solution is washed but twice with 0.25 l. of water and twice with 0.25 l. of 80 per cent methyl alcohol. This is sufficient to precipitate the chlorophyll when the methyl alcohol is completely washed from the petroleum ether. It is taken up in the usual manner with talc, washed upon the suction filter with petroleum ether and immediately extracted from the "Nutsch" with ether. The ether is dried with sodium sulphate and, after quickly concentrating, the chlorophyll is precipitated from it by means of low boiling petroleum ether. This point can be reached in 35–40 minutes.

The yield amounts to 0.25 g. while the leaves used (corresponding to 50 g. of dry leaves) contained 0.4 to 0.5 g. of chlorophyll. The preparation is free from yellow pigments.

Crude Chlorophyll from Fresh Brown Algae.[7]

The Pheophyceae can not be used in the dry condition as can the land plants.

After carefully drying and grinding brown algae, only about 5 per cent of their originally contained chlorophyll and not more than this percentage of the fucoxanthin could be extracted and even this small quantity of pigment was in no single case in an undamaged condition. It is, therefore, necessary to work up the brown algae when they are in a fresh condition, either extracting them or at least steeping them to destroy the enzymes. The difficulties that were involved in grinding and extracting the tenacious materials were overcome by a preliminary extraction with 40 per cent acetone; that is, according to our general method of working up fresh leaves, which stands the test even in the treatment of plant material so difficult to handle.

Much mucilaginous matter is extracted by this procedure, so that afterwards the algae may be easily ground and all the pigment can be extracted with acetone having a lower water content.

Fucus virsoides, from the Adriatic Sea, was chiefly worked up in this way. We are greatly indebted to the Zoological Station of the

[7] Unpublished work of R. Willstätter and H. J. Page.

Kaiser-Wilhelm Gesellschaft, in Rovigno, and its director, Dr. Thilo Krumback, for kindly supplying us with many, large quantities of Fucoideae.

The algae were quite coarsely ground, in quantities of 10–20 kg., in a machine driven roller mill and immediately placed in 40 per cent acetone (2 l. for each kg. of algae) for a period of 15 to, at most, 30 minutes, during which they were frequently stirred. They were then filtered upon a large "Nutsch" and the mucilaginous fluid was still more completely separated by means of a hydraulic press, using a pressure of 300 atmospheres. The material may now be much more finely ground between the stone rollers.

To avoid too great dilution of the extract the comminuted algae are extracted, in portions corresponding to 3 kg. of the initial material, five times with 85 per cent acetone in the following manner: the material is placed in a filtering jar, stirred up with 3 l. of the solvent and filtered under suction. The once extracted meal is placed in a second filtering jar, is mixed with a fresh 3 l. portion of acetone and again filtered under suction. The second filtrate (not the first) serves for the first extraction of the second portion of the material. The first charge of algae is put into a third filtering jar, its third extract serves for the second extraction of the second portion and so forth, till each 3 kg.-charge has been extracted 5 times. The fifth extract of each charge serves for the fourth extraction of the following charge, for the third extraction of the second following charge, and so forth. When chlorophyll-containing extracts come in contact with fresh meal the chlorophyll precipitates and only goes into solution again with a subsequent extraction. All the first extracts, consequently, are yellowish brown and almost free from chlorophyll; they are used only for the preparation of fucoxanthin. The second to fifth extracts, which are olive-green, then pure green, are united (for example, 25 l. from 20 kg.) and chlorophyll is precipitated from approximately 4 l. portions thereof by stirring up these portions with talc and carefully diluting them with the requisite amount of water. The appropriate degree of dilution so that the greater portion of the fucoxanthin will remain in solution must be found for each experiment.

In every case some of the fucoxanthin goes into the talc, which is, therefore, washed on the suction filter, first with 65 per cent acetone and then once with 60 per cent alcohol. The filtrates, collectively, serve as initial material for the isolation of fucoxanthin (Chapter XII,

section 4); the talc contains the crude chlorophyll in an approximately quantitative yield.

From this chlorophyll, diluted with talc, potassium chlorophyllin, and also pheophytin and phytol, were prepared.

The chlorophyll derivatives consist almost entirely of compounds of the *a*-series. It is, therefore, true that the brown algae may serve as a natural initial material for the preparation of the so-called blue component of chlorophyll, for which H. C. Sorby[8] first recommended them. The isolation of component *a* is so simple because Sorby's third chlorophyll component, chlorofucin, does not exist in the plant at all nor does it form under the conditions that are described here.

The yield of pheophytin from 10 kg. of Fucus that contained, according to quantitative determinations, 5 g. of chlorophyll amounted to 4.5 g. and the yield of potassium chlorophyllin was 4.2 g.

5. Description of Chlorophyll.[9]

Preparations of pure and undamaged chlorophyll have the following characteristics:

1. The ash content must amount to 4.5 per cent. and the ash must consist of pure magnesium oxide.[10]
2. The phytol content amounts to a third of the molecule and the phytol must be free from solid admixtures.
3. The chlorophyll must not contain any yellow pigments.
4. The brown phase must appear upon saponification with alkali.
5. Cleavage must produce the normal mixtures of phytochlorin *e* and phytorhodin *g*.
6. The spectrum must agree with that of the leaf extract.

Preparations made according to the new methods fulfilled these conditions and the analytical data of the work of Willstätter and Hug have been supplemented and improved by means of the new preparations.

Since no marked difference is shown between the compositions of the two chlorophyll components in consequence of the hydration of

[8] Proc. Roy. Soc. 21: 452, 1873.

[9] Ann. d. Chem. 380: 204, 1911.

[10] The ash does not contain any phosphorus; the statements of J. Stoklasa regarding the phosphorus and potash content of chlorophyll are incorrect.

chlorophyll *a*, the values for mixtures of *a* and *b* deviate but slightly from that calculated for component *a* alone.

Chlorophyll *a* (half hydrate) : $C_{55}H_{72}O_5N_4Mg + \frac{1}{2}H_2O$.

Calculated for	$C_{55}H_{73}O_{5\frac{1}{2}}N_4Mg$	Found[11]
C	73.17	73.53
H	8.15	8.09
N	6.21	6.14
Mg	2.70	2.60
OCH_3	3.44	3.33
Phytol	32.85	32.1

The following reactions were more sensitive than an analysis in deciding whether the chlorophyll is pure and whether the easily altered radicles of the molecule are undamaged.

Phase test. The ethereal solution is shaken with methyl alcoholic potash; the color changes[12] to a pure brown; then, after a few moments the original chlorophyll color returns in the alkaline medium.

The phase does not appear with allomerized chlorophyll and is muddy brown in the presence of admixtures.

If much water is added immediately at the beginning of the brown phase the greater portion returns to the ether as green pigment. The regenerated chlorophyll, strange to say, is wholly uninjured and not even allomerized; it gives the brown phase again with methyl alcoholic potash and the normal cleavage products may be obtained from it. But if water is added to the alkaline layer only after the return of the green color, the ether does not again assume a green color.

Test for the Presence of Yellow Pigments. In the phase test the ether must become colorless. But if carotin and xanthophyll are present, these, in part, pass into the alkaline layer and are transferred to the ether only by the slow addition of water; completely so upon repeated extractions of the alkaline fluid with ether.

The absence from a preparation of the yellow accompanying substances, which very persistently follow the chlorophyll, indicates, according to experience that has been acquired in the determination of the degree of purity, that this preparation is also free from colorless admixtures.

11 Unpublished.

12 This reaction was probably first observed by H. Molisch. Ber. d. d. bot. Ges. 14: 16, 1896.

Cleavage test. 3–4 cc. of boiling, concentrated methyl alcoholic potash are poured over a few milligrams of the powdered substance and the boiling is continued a few minutes, taking precaution that the solution does not boil down so far that the iso-chlorophyllins are decomposed to the simpler phyllins. After acidification the bases are extracted with 30 cc. of ether, the phytochlorin *e* is first extracted with 4 per cent HCl and then the phytorhodin *g* with 9 per cent HCl. Only a little rhodin *g* should now remain in the ether; the ether now becomes colorless on the addition of 12 per cent HCl and when the solution is neutralized and thoroughly shaken with a little ether a more concentrated, beautiful red rhodin solution is obtained. But if the preparation contains allomerized chlorophyll, feebly basic phytochorin is present and this causes the last phytorhodin fraction to be off color.

Test as to Full Phytol Content (Basicity Test). The phytol ester group may be attacked by alcoholysis or hydrolysis. In the elaboration of leaves that have a considerable chlorophyllase content, formation of some free chlorophyllide takes place when they are extracted with aqueous acetone and similarly an admixture of alphyl chlorophyllide occurs in the case of their extraction with alcohol.

Testing the ethereal solution with 22 per cent HCl, which extracts the simple chlorophyllides, is far more sensitive than a quantitative phytol determination.

The ethereal solution can be tested for free chlorophyllides with a 0.01 *N* caustic potash solution.

Test as Regards an Undamaged Magnesium Complex. If chlorophyll is injured by the action of acid, the admixture of pheophytin is betrayed in the spectrum by the appearance of absorption bands before the Fraunhofer line E and between the lines E and F.

Chlorophyll shows neither acid nor basic properties; it is sensitive to acids and alkalies.

When acted upon by acids chlorophyll immediately undergoes a change of color to olive brown and it loses its magnesium. Upon the addition of oxalic acid to its alcoholic solution, the color turns olive green for a short time and a compound precipitates in the form of olive-colored floccules which change into pheophytin only upon standing. No picrate is obtained with picric acid but the chlorophyll is decomposed and gives a brown solution.

Chlorophyll itself is a bluish-tinted, black substance, which has a strong, almost metallic, luster. It may be very easily pulverized in the dry state to a dull, greenish- tinted or bluish- tinted, black powder. Under the microscope chlorophyll appears crystalline, occasionally it even distinctly has the form of crystalline aggregates so that sharp boundaries may be seen at the edges of the aggregate. Individually formed crystals did not occur, however.

The substance does not possess a sharp melting point; the temperature of fusion depends upon the manner of heating. When slowly heated at 100°, as in drying for example, the substance sinters; it then produces when cooled a brittle, black mass whose properties are not appreciably changed. When warmed in a melting point tube the chlorophyll forms viscous drops, sometimes somewhat below 100° and sometimes somewhat above 100°; as a result of several determinations we found the melting point to be 93–96° and 103–106°.

Chlorophyll is easily soluble in absolute alcohol with a bluish-tinted green color, somewhat more difficultly so in 95 per cent alcohol as well as in methyl alcohol and difficulty so in 90 per cent methyl alcohol. In contrast with ethyl and methyl chlorophyllides, it dissolves very easily in ether and gives a beautiful, blue green solution, which fluoresces strongly; it has a more bluish tint than the alcoholic solution. Its behavior towards petroleum ether is surprising; chlorophyll is extraordinarily difficultly soluble in it in the cold, when warm it is slightly soluble. Upon the addition of a little methyl or ethyl alcohol it is, on the contrary, easily soluble. Hexane from petroleum (Kahlbaum) dissolves chlorophyll rather easily; petroleum ether precipitates it from this solution; in hexane from propyl iodide, on the other hand, it shows just as great insolubility as in petroleum ether.

Benzol dissolves it very easily; cyclohexane, chloroform and carbon disulphide easily, the last with a green color that is less blue than in the case of the other solvents. The substance is very easily soluble in pyridine.

Fractionation According to the Method of Kraus. Isolated chlorophyll, in spite of its insolubility in pure petroleum ether, shows the same distribution between petroleum ether and alcohol that has been known to occur in the case of the pigment of leaf extracts since the fundamental experiments of Stokes and Kraus; this behavior is a result of its solubility in alcohol-containing petroleum ether. When subjected to fractionation with a little water there results an approxi-

mately equal distribution of the pigment between the two layers. Upon the addition of much water or upon repeated shaking out with pure petroleum ether the aqueous, alcoholic layer retains only a very pale, yellowish green color.

The fractionation ratios for 0.1 per cent ethyl and methyl alcoholic solutions have been ascertained by colorimetrical determinations.

(a) 25 cc. of petroleum ether is added to 25 cc. of an alcoholic solution which is then fractionally separated by means of 5 cc. of water; the two layers appear to be approximately the same. According to the colorimetric determination the petroleum ether contains 44 per cent of the pigment.

(b) In a similar experiment, using methyl alcohol, the petroleum ether solution contains 56 per cent of the chlorophyll. The methyl alcoholic layer is again treated with 25 cc. of petroleum ether; it extracts the pigment almost quantitatively from the lower layer.

No crystals separate from the petroleum ether when it stands over the dilute alcoholic layer, which is in contrast to the experiment with ethyl chlorophyllide.

Alteration in Alcoholic Solution (Allomerization)[13]

Chlorophyll in alcoholic solutions is subject, especially in anhydrous solvents, to a transformation that has made its isolation and purification extraordinarily difficult. The simple alphyl derivatives also undergo this transformation, the free chlorophyllides especially quickly. The chlorophyllides, as a result, lose their power of crystallization, they no longer show the brown phase and they produce weakly basic cleavage products instead of the normal ones. We have designated this transformation an allomerization,[14] and attempt to explain it as the opening of lactam groups and the formation of new ones.

To ascertain the cause of the allomerization and to prevent its appearance was of great practical importance and experiments were carried out chiefly with methyl chlorophyllide for this purpose. Chlorophyllides in alcoholic solution are protected to some extent by water, even by 2 per cent of water, but aqueous solvents do not sufficiently dissolve the alkyl chlorophyllides.

[13] Ann. de Chem. 382: 135. 1911, and 387: 325 and 357. 1912.

[14] The name is derived from the fact that in this phenomenon the radicals of the molecule become rearranged. Since the composition of the altered (allomerized) chlorophyllide is not exactly known, it is not proper to speak of it as an isomer.

A solution of methyl chlorophyllide in absolute alcohol gave, after 10 hours, only a slight color change on the addition of caustic potash and, after 24 hours, none; upon the addition of 5 per cent of water the brown phase was still very distinct after 2 days and it did not disappear until after 5 days.

The transformation does not take place in ether, chloroform or anhydrous pyridine; ethereal solutions of phytyl and methyl chlorophyllide *a* remained unchanged for 3 weeks even when in glass vessels.

Allomerization in an alcoholic solution is promoted catalytically by glass. It does not occur in platinum or silver vessels.

A solution of methyl chlorophyllide *b* in absolute alcohol had not changed at all after 5 days in a platinum test-tube while, after 24 hours in a glass vessel excluded from the influence of light, it no longer gave the brown phase and showed a dull green color.

This phenomenon, however, cannot be attributed simply to the alkalinity of the glass, for allomerization occurred similarly in a test-tube of pure quartz. (Methyl chlorophyllide *b*, 0.001 mole in 1 l. of ethyl alcohol, had allomerized almost quantitatively in it after 24 hours, somewhat more slowly than in glass.) Methyl chlorophyllide *b*, dissolved in absolute alcohol in a platinum vessel, was not perceptibly altered after 24 hours by 0.000,001 *N* KOH while, on the contrary, the greater portion of that in a quartz flask had been transformed.

Even if allomerization cannot be explained simply as a catalysis by small amounts of alkalies we have nevertheless succeeded in preventing it by the addition of exceedingly small amounts of acid. To a solution of methyl chlorophyllide in absolute alcohol, for example, we add a trace of alcoholic, oxalic acid solution. The color and the phase remained unchanged after several days, even after 3 weeks; a control experiment in pure alcoholic solution did not give the phase after even one day; in a second comparison experiment with the addition of a trace of potassium hydroxide, the phase did not appear even after a few hours.

The formerly puzzling phenomenon, that chlorophyll and chlorophyllides show a great tendency toward alteration in their pure solutions and not in their extracts, is now explained. The extracts contain water and, frequently, traces of organic acids.

By means of the protection of very small quantities of acid, suitably 0.01 g. of oxalic acid in 1 l. of alcohol, the treatment of the sensitive magnesium compounds, especially the separation into components

which is described in Chapters VI and X, is much facilitated and the isolation of the pure substances is made more certain.

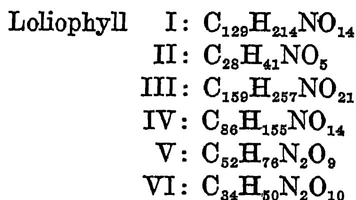
6. Comparative Investigation of the Chlorophyll from Different Plants.[15]

A knowledge of the characteristics and composition of chlorophyll furnished the basis for the comparison of the chlorophyll from different plants.

Former views regarding the identity or dissimilarity of the chlorophyll from different plants differed greatly and were not based upon chemical analysis.

A. Gautier,[16] in his treatise, "Sur la Pluralité des Chlorophylles," considered the chlorophyll in monocotyledons and dicotyledons to be different without, however, making any statements regarding the differences, and he even emphasized its diversity "dans le même embranchement."

Even in 1906, A. Étard,[17] in his book "La Biochimie et les Chlorophylles," described an endless number of chlorophylls which were not altogether pigments from different sources; for example, for the single plant, Lolium perenne, he set up a whole series of different chlorophylls whose formulae



Loliophyll I: $C_{129}H_{214}NO_{14}$
II: $C_{28}H_{41}NO_5$
III: $C_{159}H_{257}NO_{21}$
IV: $C_{86}H_{155}NO_{14}$
V: $C_{52}H_{76}N_2O_9$
VI: $C_{34}H_{50}N_2O_{10}$

differ in an unparalleled manner. They are absurd; each fraction of more or less green colored wax was considered an individual chlorophyll.

Proof of the identity of chlorophyll from any source is revealed by the determination of its basic cleavage products, phytochlorin and phytorhodin, the determination of its magnesium content, and the determination of its phytol content.

After success was attained in avoiding alterations of the pigment during its isolation from plants, the chlorophyll from all plants yielded

[15] Papers VII, X, XIV.

[16] Compt. rend. 120: 355. 1895, and Bull. soc. chim. (4) 5: 319. 1909.

[17] Masson and Co., Paris.

the same nitrogen-containing derivatives, the determination of which is discussed in Chapter IV.

No variations appear, therefore, in the composition of the phytochromin.

Now, it was still conceivable that other elements besides magnesium could appear in the rôle of that complexly bound metal. It was, therefore, not sufficient to prepare and compare the metal-free, decomposition products from different plants; it was also necessary to analyze preparations either of chlorophyll itself or of a chlorophyllide or, just as well, preparations of a crystallized phyllin, from the most varied sources possible. This investigation was carried out by Willstätter and Pfannenstiel,[18] who obtained the same rhodophyllin, of the formula, $C_{33}H_{34}O_4N_4Mg$, with an ash content of 7 per cent MgO, from plants of the following groups:

Cryptogams: Chlorophyceae,
Musci, Filicales, Equisetales;
Phanerogams: Monocotyledons; Gramineae,
Dicotyledons; Urticaceae, Saxifragaceae.

This comparative investigation of the magnesium content was afterwards still further expanded and supplemented by the isolation of chlorophyll, or of its immediate magnesium-containing derivatives, from a greater number of plants of other orders (for example, from the Pheophyceae).

The third point included in our comparison has to do with the nitrogen-free alcohol whose distribution in nature is extraordinary. It would have been too unsafe to derive from a few examples the proposition that phytol is a constituent of the chlorophyll from all plants and that it always amounts to a third of the molecule. This alcohol did not seem to be irreplaceable by other alcohols to the same extent as was magnesium by other metals. We, therefore, used over 200 genera of plants from the various classes for the isolation of phytol by the saponification of pheophytin and for the determination[19] of the phytol number. In addition, phytol was identified by the elementary analysis of preparations from 24 plant species.

As a consequence of the action of chlorophyllase, the phytol number was found to fluctuate in our first experiments. After this source of

[18] Paper V.

[19] Ann. d. Chem. 378: 1. 1910.

error was ascertained it was possible, by extracting the leaves as quickly as possible, to establish the *constancy of the phytol number.* No exception as regards the phytol content was observed.

The examples were so chosen that plants subject to the most varied living conditions came under observation: sea algae, fresh-water plants and tropical plants in addition to indigenous land plants.

Of the cryptogams, the following classes (with 12 examples) were represented:

Chlorophyceae, Charales, Pheophyceae, Bryophyta and Pteridophyta (namely, Filicales, Equisetales and Lycopodiales).

The gymnospermae (2 examples of conifers) and monocotyledonae (24 examples) were from the following families:

Potamogetonaceae, Gramineae, Palmae, Araceae, Liliaceae and Musaceae.

Finally there were 164 examples of dicotyledons, from the families:

Salicaceae, Juglandaceae, Betulaceae, Fagaceae, Ulmaceae, Moraceae, Urticaceae, Aristolochiaceae, Polygonaceae, Chenopodiaceae, Ceratophyllaceae, Ranunculaceae, Magnoliaceae, Lauraceae, Papaveraceae, Cruciferae, Saxifragaceae, Platanaceae, Rosaceae, Leguminosae, Simarubaceae, Polygalaceae, Linaceae, Buxaceae, Celastraceae, Aceraceae, Hypocastanaceae, Balsaminaceae, Tiliaceae, Malvaceae, Bombacaceae, Dilleniaceae, Guttiferae, Dipterocarpaceae, Violaceae, Passifloraceae, Lecythidaceae, Oenotheraceae, Hallorrhagidaceae, Araliaceae, Umbelliferae, Cornaceae, Primulaceae, Oleaceae, Loganiaceae, Gentianaceae, Apocynaceae, Convolvulaceae, Borraginaceae, Verbenaceae, Labiatae, Solanaceae, Scrophulariaceae, Bignoniaceae, Acanthaceae, Plantaginaceae, Rubiaceae, Caprifoliaceae, Valerianaceae, Dipsacaceae, Cucurbitaceae, Campanulaceae, Compositae.

VI. ISOLATION OF THE TWO COMPONENTS OF CHLOROPHYLL.

1. History of the Method.[1]

Stokes

G. G. Stokes discovered that chlorophyll consists of two components. His announcement, which appeared in the year 1864 in the valuable note,[2] "On the Supposed Identity of Biliverdin with Chlorophyll, with remarks on the Constitution of Chlorophyll," reads:

"I find the chlorophyll of land-plants to be a mixture of four substances, two green and two yellow, all possessing highly distinctive optical properties. The green substances yield solutions exhibiting a strong red fluorescence; the yellow substances do not. The four substances are soluble in the same solvents, and three of them are extremely easily decomposed by acids or even acid salts, such as bisoxalate of potash; but by proper treatment each may be obtained in a state of very approximate isolation, so far at least as colored substances are concerned."

The investigation of Stokes, therefore, consisted in the spectroscopical differentiation of the two components of chlorophyll and of the accompanying yellows, carotin and xanthophyll.

The method by which the great physicist separated the mixture of pigments that is contained in the leaf extract is revealed by a single remark in the lecture,[3] "On the Application of the Optical Properties of Bodies to the Detection and Discrimination of Organic Substances:"

"For convenience and rapidity of manipulation, especially in the examination of very minute quantities, there is no method of separation equal to that of partition between solvents which separate after agitation . . . Bisulphide of carbon in conjunction with alcohol

[1] Ann. d. Chem. 390: 275. 1912.

[2] Proc. Roy. Soc. 13: 144. 1864. See Stokes' supplementary footnote in the reprint of the noted treatise of the year 1852: "On the Change of Refrangibility of Light." Mathematical and Physical Papers, Vol. III, p. 300, Cambridge, 1901.

[3] Journ. Chem. Soc. 17: 304, 311. 1864.

enabled the lecturer to disentangle the colored substances which are mixed together in the green colouring-matter of leaves.''

Stokes, therefore, first used the fruitful method of fractionation to separate chlorophyll from the yellow pigments and to separate the chlorophyll into its components. Unfortunately, neither Stokes's proposed treatise on chlorophyll appeared nor did the publication of his posthumous scientific writings bring to light notes on the subject.

Kraus and Sorby.

G. Kraus[4] knew the results of Stokes that had been published in the Proceedings of the Royal Society, but not his method, and, independently of Stokes, he discovered the fractionation of a crude, alcoholic, chlorophyll solution by means of benzol. A little later R. Sachsse[5] used benzine instead of benzol. Kraus attained by the fractionation method a separation into green and yellow pigments only and did not develop the method for the resolution of the so-called cyanophyll and xanthophyll into their two components. It was only in a discussion of the investigation of Sorby, which had appeared a short time previously, that Kraus alluded to the composition of the yellow and green constituents as consisting of two components each and he was not able to confirm this.

H. C. Sorby states[6] that he was incited to his extensive, spectroscopial investigations by a lecture of Stokes[7] delivered in the Royal Institution and that he had, as a result of independent experiments, come to the same conclusions as had Stokes previously; it is not probable, however, that Sorby discovered the fractionation method uninfluenced by the publication of Stokes. Sorby aimed at the fractional separation of the pigments of a crude chlorophyll solution by means of their systematic distribution between aqueous alcohol and carbon disulphide for the sole purpose of the spectroscopic differentiation and description of the fractions. These, however, were not as yet optically homogeneous, not mentioning the fact that the solutions also contained colorless accompanying materials. Sorby's spectro-

[4] Zur Kenntnis der Chlorophyllfarbstoffe und ihrer Verwandten. Stuttgart. 1872.

[5] Die chemie und Physiologie der Farbstoffe, Kohlenhydrate und Proteinsubstanzen. p. 23. Leipzig. 1877.

[6] On Comparative Vegetable Chromatology, Proc. Roy. Soc. 21: 442. 1873; see also Proc. Roy. Soc. 15: 433. 1867; Quarterly Journ. of Microscopical Science 11: 215. 1871; Quarterly Journ. of Science 8: 64. 1871.

[7] Proc. Roy. Soc. 15: 433. 1867 and 21: 451. 1873.

scopical data were not as yet exact and have proved to be only partially correct.

Besides the yellow pigments, Sorby distinguished between three different chlorophyll pigments which showed absorption at both ends of the spectrum and exhibited a red fluorescence; blue chlorophyll, yellow chlorophyll and chlorofucin.

The application of fractionation acquires an entirely different import in the work of N. A. Monteverde.[8] There appears here the question of the different distribution of amorphous and crystallized chlorophyll between alcohol and petroleum ether. The observations of Monteverde are explained by the more exact knowledge that has been acquired on Borodin's chlorophyll. Upon fractionation, the phytol-containing chlorophyll passes into the layer of petroleum ether and the alcoholized, phytol-free chlorophyll enters the alcoholic layer.

W. H. Hartley[9] thought that he had succeeded in separating blue from yellow chlorophyll by the use of a new method. He allowed a warm, saturated barium hydroxide solution to act upon the alcoholic solution of crude chlorophyll.

Boric acid in glycerin liberates Hartley's blue chlorophyll from the precipitated barium compound while the filtrate from the barium compound contains the yellow chlorophyll.

As has already been shown, however, chlorophyll is saponified by barium hydroxide;[10] if the saponification has not been sufficiently energetic there remains in the filtrate from the barium chlorophyllin mostly yellow accompanying substances, mixed with a little green pigment.

Tswett.

M. Tswett[11] appreciated the meritorious observations of Stokes and confirmed them by means of a new efficient method. Tswett separated

[8] Das Absorptionsspektrum des Chlorophylls, Acta Horti Petropolitani XIII, 123. 1893.

[9] Journ. Chem. Soc. 59: 106. 1891 and 85: 1607. 1904.

[10] Ann. d. Chem. 350: 63. 1906.

[11] Physikalisch-Chemische Studien über das Chlorophyll. Die Adsorptionen. Ber. d. d. bot. Ges. 24: 316. 1906. Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemie des Chlorophylls, Ibidem 224: 384. 1906. Spektral-analytische Untersuchungen über die Chlorophylline und deren nächste Säurederivative (Chlorophyllane). Ibidem 25: 137. 1907. Über die Spektrophotometrie der Chlorophylline und die Energetik des Chlorophylls. Ibidem 25: 388. 1907. See also Ber. d. d. Chem. Ges. 41: 1352. 1908; 43: 3199. 1910; 44: 1124. 1911 and the Russian book: Chromophyll in the plant and animal world. Warsaw, 1910 (by Karbasnikow).

the mixture of pigments that is contained in leaf extracts by means of chromatographic adsorption analysis into its individual components and carefully investigated the spectra of these.

The principle of the method consists in the fact that pulverulent substances adsorb pigments, as well as colorless bodies, to different extents from their solutions in organic fluids. Tswett uses a solution of crude chlorophyll in certain solvents, petroleum ether, benzol and carbon disulphide, for this analysis and filters it through a column of calcium carbonate, inulin or sugar. The pigments are precipitated in this way and they mutually displace each other from their adsorption compounds in the decreasing order of their action upon the surface tension of the solvent, so that in the resulting "chromatogramm" there appears a stratification into as many different colored zones as there are components present in the mixture of pigments. The pigmented column may be systematically separated with a scalpel and each individual pigment may be extracted again with suitable solvents—alcohol, ether or chloroform.

By this method Tswett finds five yellow pigments (carotinoids) in the mixture, namely, carotin and four xanthophylls and two real chlorophyll pigments which he calls α and β chlorophyllin (corresponding to what we designate chlorophyll components *a* and *b*). Tswett has described spectroscopically the solutions of these components; his statements, so far as they concern the green components, have been confirmed in all essentials by our investigations.

The chromatographic method has as yet been used on a small scale only and it appears unsuitable for preparative work. How far Tswett succeeds, in the formation of the chromatogram and the isolation of the chlorophyll components from the same, in preventing[12] the allomerization of chlorophyll is as yet unknown; this can not be settled by a spectroscopical investigation but only by testing the phytochlorin and the phytorhodin.

2. Procedure of Willstätter and Isler.[13]

The observations of Stokes, Sorby and Tswett on the fractionation of chlorophyll agree essentially; a part of these data; namely, the observations on the pigments of the brown algae, has been corrected

[12] Alcohol must not be used in extracting the pigment from the chromatogram; See R. Willstätter and A. Stoll. Ann. d. Chem. 387: 357. 1912.

[13] Paper No. XX.

as a result of our subsequent tests (Chapters IV and V). It has, up to the present time, been impossible to determine whether the results of the fractionation refer to the natural pigment or whether they are dependent upon changes in the chlorophyll during extraction and fractionation. The refutation of the data on the Pheophyceae pigments particularly shows this clearly. Detection of changes in the chlorophyll is made possible only by the investigation of its cleavage products, the phytochlorins and the phytorhodins, especially by their fractionation according to the method of Willstätter and Mieg. The investigation of absorption spectra, the only method available to the earlier authors, is, on the contrary, not adapted to the recognition of decided changes, such as allomerization.

The question whether chlorophyll was made up of two components was, therefore, ready for solution only after the chemical characteristics of chlorophyll and its relations to its decomposition products were determined.

In the preparation of pure chlorophyll by Willstätter and Hug's method the question whether chlorophyll is a homogeneous substance or a mixture of two components was not considered at any time. After the isolation of the green pigment in the same combination in which it occurs in the green plants had been accomplished the method of the distribution of chlorophyll between several non-miscible solvents established, in Willstätter and Isler's continuation of this investigation, that a blue green component (*a*) and a yellow green (*b*) exist in the extracts just as in the leaves. Systematic fractionation by the method of fractional distribution in non-miscible solvents led to the gradual displacement of the component ratio, finally to such an extent that perfectly homogeneous components were obtained from the mixture.

The two chlorophyll components were obtained entirely free from colorless and colored accompanying materials and, what was much more difficult to attain, free also from allomeric compounds which form spontaneously and with extreme ease.

In the isolation of chlorophyll by Willstätter and Hug's method the pigment of the extract was separated into three fractions:

Methyl alcoholic wash liquors,
petroleum ether residual solution and
the main methyl alcoholic solution.

Although the main solution does not show any considerable displacement of the component ratio, we find:

1. The component *b* greatly predominates in the methyl alcoholic wash liquor, especially in the very first extracts with methyl alcohol.
2. The component *a* is almost quantitatively present in the residual petroleum ether solution and is contaminated with only a small amount of *b*.

Since the two purifying operations displace the component ratio in opposite directions, it is quite comprehensible why this remains rather unchanged in spite of the often repeated distribution of the chlorophyll between different solvents.

Upon this observation of the distribution of the two components between methyl alcohol and petroleum ether is founded our method for their complete separation; by means of the systematic fractionation of the chlorophyll mixture between aqueous methyl alcohol and petroleum ether the *b* component accumulates in the methyl alcohol and the *a* component in the benzine layer and they are finally obtained in the pure state.

This method was very simply modified so that the petroleum ether solution of crude chlorophyll, prepared according to Willstätter and Hug's method, was washed frequently (14–16 or even 20 times) with 90 per cent methyl alcohol; the petroleum ether was then free from chlorophyll *b*. The chlorophyll in this layer had attained a sufficiently high degree of purity to be precipitated by completely washing out the methyl alcohol. It is worth while to work up for the *b* component the first six methyl alcoholic wash liquors; the chlorophyll *a*, which has passed into these liquors in considerable amounts, can be almost wholly removed from them, of course with considerable loss in *b*, by washing them at least three times with petroleum ether. After diluting with water, the component *b* is then also transferred to petroleum ether and precipitated by Willstätter and Hug's method from the petroleum ether layer by washing out the methyl alcohol.

Although this simple principle aids in the separation of the two components the method nevertheless is not sufficient for their preparation in a wholly pure state.

The component *b* contains persistent admixtures of xanthophyll, which are betrayed by the cleavage test.

The component *a*, to be sure, is free from *b* and from yellow pigments but it is apparently not wholly free from colorless accompanying substances; under these circumstances it precipitates in such a fine, flocculent form that filtering is almost impossible (without diluting with talc or the like).

By frequent extraction of the petroleum ether layer with 90 per cent methyl alcohol the degree of purity of the chlorophyll that remains is not continually increased but, as Willstätter and Hug have found, a point is reached after several extractions where the chlorophyll that passes into the methyl alcohol possesses a higher degree of purity than that remaining in the petroleum ether.

It is highly advantageous, therefore, in the purification to extract the chlorophyll with aqueous methyl alcohol; that is, with the same solvent with which it was previously washed and then bring the chlorophyll from this into petroleum ether again before attempting its separation.

In this method of isolation, however, it is disadvantageous to remove and reject, in the process of the separation of component *b*, very much of the highly pure *a*. The process described is, consequently, considerably improved by not undertaking the separation of the two components until the mixture in its petroleum ether solution has been washed with 90 per cent methyl alcohol and then brought to a high degree of purity by extraction with 95 per cent alcohol.

This method of operation has still another important advantage. If the original petroleum ether solution is washed several times with 90 per cent methyl alcohol and these extracts are rejected the xanthophyll is quantitatively removed by this procedure and the component *b* may be isolated in a strictly pure state even though it be with great loss.

The method was perfected in the course of 74 experiments to such an extent that it gave, in a working day (12 hrs.) with a charge of 4 kg. of dry leaves, yields of 1–1.25 g. of chlorophyll *a* and 0.1–0.4 g. of chlorophyll *b*, and, in addition, yielded the two pheophytin components as by-products.

3. The Chlorophyll Components by the Method of Willstätter and Stoll.[14]

Our new method has this principle in common with the method of Willstätter and Isler: the distribution of chlorophyll between methyl

[14] Unpublished.

alcohol and petroleum ether in which the *a* component prefers the petroleum ether phase and the *b* component the methyl alcoholic phase. Instead of proceeding from the extracts, isolated chlorophyll, which has been made much more easily obtainable, is used as initial material for the fractionation. Larger quantities and more concentrated solutions can then be used and the method can be made almost quantitative.

If the component *b* is extracted with aqueous methyl alcohol from the petroleum ether solution of pure chlorophyll a certain concentration of the pigment must not be exceeded in order to keep the portion that remains in the petroleum ether in clear solution. The initial concentration may amount to 2 g. of chlorophyll in 1 l. of petroleum ether. According to preliminary experiments, 85 per cent methyl alcohol is most suitable for the fractionation; 80 per cent extracts too little of the pigment; too much of the component *a* would be extracted with the *b* in 90 per cent methyl alcohol.

The distribution of chlorophyll between aqueous methyl alcohol and petroleum ether under those conditions that are of practical interest have been approximately determined[15] in the following experiments by the methods of Chapter IV.

One gram of chlorophyll, of component ratio 2.7, is dissolved[16] in 1 l. of petroleum ether and extracted with half its volume of 85 per cent and later 90 per cent methyl alcohol, which has been previously saturated with petroleum ether.

It is seen that the following portions pass from the petroleum ether (0.64–0.66) into an equal volume of methyl alcohol:

of *a* into 85 per cent CH_3OH, 3 per cent; into 90 per cent, 8–9 per cent;

of *b* into 85 per cent CH_3OH, 17–18 per cent; into 90 per cent, about 60 per cent;

distribution ratio for petroleum ether and 85 per cent CH_3OH: *a*, 32; *b*, 4½;

distribution ratio for petroleum ether and 90 per cent CH_3OH: *a*, 11; *b*, 2/3.

[15] For the determination the extracts were transferred to ether, evaporated and an aliquot portion of this was then saponified with methyl alcoholic potash.

[16] The solution is prepared by dissolving the chlorophyll in 25 cc. of ether which, after mixing with petroleum ether, is subsequently removed by washing with 50 per cent. methyl alcohol.

From these determinations it may be concluded that, for the isolation of chlorophyll *b*, only extracts with a component ratio of less than one to one are suitable and that it is expedient, in order to free the methyl alcoholic solution of component *b* from *a* by washing with petroleum ether, to increase the concentration of the methyl alcohol; for example, to 90, but not more than this, and, in consideration of the very variable distribution ratios, to wash with but little petroleum ether.

Procedure.

Eight grams of chlorophyll are dissolved in 150–200 cc. of ether and the opaque fluid, in order to be sure that it contains no undissolved

Solvent	Extract	Per cent of original component *a*	Per cent of original component *b*	Component ratio
85% CH_3OH	1 + 2	3.4[17]	16.1	0.56
"	3 + 4	2.9	14.8	0.52
"	5 + 6	2.8	13.2	0.58
"	7 + 8	2.8	11.4	0.72
"	9 + 10	3.0	9.6	0.84
"	11 + 12	3.0	7.8	1.02
"	13 + 14	2.8	6.3	1.19
"	15 + 16	2.7	5.0	1.44
90% CH_3OH	17 + 18	6.8	8.0	2.23
"	19 + 20	5.7	4.0	3.81

particles, is filtered into a 7 l. separatory funnel which contains 4 l. of petroleum ether (0.64–0.66). Ordinarily the chlorophyll begins to precipitate again when this is done and an addition of 50–100 cc. of methyl alcohol is required to clear the solution.

The ether must be removed prior to the fractionation by washing with 2 l. of 80 per cent methyl alcohol in 1 to 2 extractions, which are discarded. When crude chlorophyll is used for the isolation of the pure components, the yellow pigments and colorless admixtures can be removed at the same time by means of these or a few more extractions.

Prior to the experiments the 85 per cent and 90 per cent methyl alcohols are saturated with petroleum ether, of which they take up 5.5

[17] This number is too high because of an admixture of ether.

and 10 per cent (respectively), and, immediately before their use, they are acidified with 0.01 g. of oxalic acid per liter.

The component *b* is sufficiently extracted by approximately 14 extractions, each with 2 l. of 85 per cent methyl alcohol; the chlorophyll of these extracts is worked up for component *b* only; that remaining in the petroleum ether, for component *a* only.

The first extract, after the separation of the petroleum ether, is brought to a concentration of 90 per cent by the addition of 1 l. of methyl alcohol, then washed thoroughly with 1 l. of petroleum ether, introduced at once into 2 l. of ether and fractionally separated by the addition of considerable water.

The second extract is also mixed with 1 l. of methyl alcohol and shaken with the wash petroleum ether from the first extract with the addition of a further half liter of petroleum ether. The purified *b* solution is then introduced into the first ethereal extract to which another liter of ether is added. These large amounts of ether are necessary because the aqueous methyl alcohol removes considerable ether and the petroleum ether which separates on dilution renders the transfer from methyl alcohol into ether difficult. Each portion of wash petroleum ether is quickly freed in a separatory funnel from methyl alcohol by means of a stream of water running through it, whereupon the pigment precipitates in a finely divided condition.

Extracts 3 and 4 are similarly purified and worked up; the content of *b* is considerably less in these.

In the usual mixtures with the component ratio 2.5 to 2.8 there is added to the sixth methyl alcoholic extract, before washing with petroleum ether, only 900 cc. of methyl alcohol, to the seventh 800, to the eighth 700, and finally to the fourteenth only 100 cc.

They are purified in pairs with the same liter of petroleum ether, to which another half liter is added when it is used the second time, and all the extracts are transferred to the same ethereal solution with the addition of more ether to this each time. At first each addition is 1 liter and then, from about the 10th extract on, each addition is 0.5 liter.

With initial material that is rich in *b* even the 6th or 7th extract is brought to a methyl alcoholic concentration of 90 per cent and it is only in the subsequent extractions that the addition of methyl alcohol is diminished about 100 cc. each time.

The sole purpose then of the 15th and the 16th extracts is to free chlorophyll *a* from the last traces of component *b*; this purification of

the petroleum ether layer is completed by shaking three more times with 90 per cent methyl alcohol, using 2 liters each time. The pigment is transferred from the methyl alcoholic wash liquors into petroleum ether; it is rich in *a* and is isolated as a by-product in the same manner as is the chlorophyll (which is relatively rich in the *b* component) of the previous petroleum ether washings.

After the separation of *b*, the blue green solution of component *a* is washed with water till the chlorophyll is quantitatively precipitated and it is then mixed with 30–100 g. of talc, depending upon its condition, in order that it may be filtered upon the "Nutsch" through a layer of talc while using weak suction. The petroleum ether runs off colorless. The talc layer is subsequently washed with low boiling petroleum ether and suction is applied till the smell of petroleum ether disappears. The pigment is then extracted from the talc by shaking in a flask with the least possible quantity of pure ether; the beautiful deep blue solution is filtered upon a small "Nutsch." The filtrate is freed from talc particles by repeated filtrations. Finally, the ether is almost completely evaporated and the concentrated solution is then washed into a dish and the ether is completely evaporated in a vacuum desiccator.

Chlorophyll *a* remains as a beautiful blue black, shining, laminated mass. The pure yellow phase with methyl alcoholic potash, the absence of yellow pigments, the quantitative formation of chlorin *e* (it is only in very large samples that phytorhodin and chlorin *g* can be detected and then both are present in traces only) and the indifference towards 23 per cent hydrochloric acid establish the purity of the preparation.

The component *b*, extracted by ether from the methyl alcoholic extracts that have been washed with petroleum ether, is in the ether-petroleum ether solution. This is freed from methyl alcohol by washing with water and, after drying with sodium sulphate, it is evaporated to about 0.5 l. The boiling point increases, as a consequence of the accumulation of the more difficulty volatile hydrocarbons, to 50–60° C.; evaporation is for this reason continued under diminished pressure at 40–50° to about 30 or 40 cc. and most of the chlorophyll *b* is then precipitated with 300 cc. of petroleum ether (B. P. 30–50°). The precipitate is filtered at once on a little talc; the mother liquor contains chiefly, as may be recognized from the brown phase, component *a*. The precipitated chlorophyll also continues to show a little of the com-

ponent *a* and must therefore be reprecipitated once (in working up initial material rich in *a*, even two or three times) from ether with petroleum ether. A little of the easily soluble *a* remains in the filtrate after each reprecipitation. The precipitate, therefore, is washed with very volatile petroleum ether, dried by suction, and again extracted with ether which is evaporated to 10 cc. and precipitated with 400–500 cc. of petroleum ether.

Component *b* has the property of precipitating in a form that filters much better than does *a*; the precipitated granules settle quickly, decantation may be used with them and they can be filtered with suction upon a hardened filter-paper. Chlorophyll *b* forms a brittle, greenish black mass after drying in a desiccator. As determined by the phase test (dark red), the cleavage (rhodin *g* only) and the basicity (23 per cent hydrochloric acid is not colored) our preparations were undamaged and free from other components.

The yield (from 8 g. with the component ratio 2.8) amounted to, for example, 3.7 g. chlorophyll *a* and 1.15 g. *b*, while 2.3 g. of a chlorophyll mixture was recovered as a by-product. Another preparation, used as initial material and obtained from fresh leaves, furnished, by way of example, from 8 g., 4 g. of component *a*, 1.2 g. *b*, and 1.5 g. of recovered mixture.

4. Description of the Chlorophyll Components.[18]

Chlorophylls *a* and *b* are microcrystalline when precipitated from ether with petroleum ether. Upon the slow evaporation of solutions in mixed ether-petroleum ether, the component *a* in particular crystallizes in characteristic forms, in aggregates of thin, lancet-shaped plates. Chlorophyll *a* forms a blue black, friable powder which gives a greenish streak and assumes a steel-blue luster when rubbed upon a smooth surface; the powder of *b* is dark green to greenish black.

In a melting point tube *a* frits and fuses to a viscous mass at 117–120°; *b* sinters between 86 and 92°; it becomes viscous at 120–130° and then begins to intumesce.

The chlorophyll component *a* is very easily soluble in ether and absolute alcohol, only moderately so in cold methyl alcohol and rather easily in warm methyl alcohol. It is easily soluble in 95 per cent ethyl alcohol, difficultly so in 80 per cent and it dissolves with difficulty in 90 per cent methyl alcohol even when warm. It is almost insoluble

[18] Ann. d. Chem. 390: 327. 1912.

in 80 per cent methyl alcohol. Chlorophyll *a* is easily soluble in acetone, chloroform and carbon disulphide, and it is also very easily soluble in benzol. The substance dissolves in petroleum ether with difficulty, even when warm. Ligroin (Kahlbaum) dissolves it somewhat better, even quite easily when warm and the solution is precipitated by petroleum ether. A very small addition of an alcohol to the petroleum ether increases the solubility of chlorophyll extraordinarily.

The ethyl alcoholic solution is blue green with a dark red fluorescence; in deep layers the light that is transmitted is ruby red. The concentrated, ethereal solution may be called pure blue; on dilution it becomes more greenish tinted. The alcoholic solution assumes a more bluish tint on mixing with petroleum ether, but not to the same degree as with ether. The color in carbon disulphide is conspicuously yellowish.

By rapid dilution of a concentrated solution of chlorophyll *a* in alcohol or acetone with much water there results a colloidal solution that is stable for weeks; it is pure green by transmitted light and does not fluoresce, but it shows a very beautiful blue green opalescence; this solution gives up its pigment to ether only after persistent, vigorous shaking, the aqueous layer becoming blue green, which it does for the first time immediately upon the addition of a little calcium chloride.

The solubility of chlorophyll *b* is, in general, somewhat less than that of *a* but the difference is very marked in petroleum ether only. The component *b* is, in fact, wholly insoluble in petroleum ether at room temperature and the solvent becomes scarcely colored, even on boiling; ligroin (also that purified with nitrosyl-sulphuric acid) dissolves the substance moderately.

The color in various solvents does not differ nearly as much as with *a*, the ethereal solution is bright green, the alcoholic somewhat duller green; solutions of *b* appear decidedly yellowish only when compared with those of *a*. The solution in carbon disulfide alone is decidedly yellowish green. Solutions in deep layers are not pure red by transmitted light but more greenish brown red; the fluorescence of component *b* is a brownish tinted red.

The colloidal solution is yellowish green by transmitted light and has a dark olive green opalescence.

Chlorophyll *b* is very easily soluble in absolute alcohol and in ether; rather difficultly so in methyl alcohol at room temperature, but quite easily on warming; 90 per cent ethyl alcohol dissolves it with more

difficulty, 80 per cent with considerable difficulty; likewise 90 per cent methyl alcohol. The substance dissolves very easily in chloroform, carbon disulphide and acetone, easily in benzol; quickly in pyridine. A benzol solution in the sunlight acquires a beautiful, red color before it gradually bleaches.

Chlorophyll component *a*, in an ethereal solution, is gradually decomposed on shaking with 6 per cent hydrochloric acid, instantaneously with 20 per cent; the cleavage takes place with somewhat more difficulty in the case of the *b* component. With an excess of ethereal hydrochloric acid the pretty blue (in the case of *b*, the green) color of a pheophytin hydrochloride appears at once.

The color of component *a* changes to a pure yellow in the phase test. Since the phase is somewhat more transient with the phytol compound than with the methyl derivatives the test in this case is generally carried out so that the ethereal solution of chlorophyll is carefully underlaid with the methyl alcoholic potash. A zone of the characteristic color then appears at the surface of separation and is immediately imparted to the whole solution on shaking. If water is added after the chlorophyll color returns, the ether remains colorless.

Chlorophyll *b* shows a bright red phase which then passes in a few minutes, but much more slowly than with chlorophyll *a*, through a motley brownish color back to the original color.

The principal absorption band in the red or orange, which is characteristic for all the chlorophyll derivatives previously investigated, is lacking in the spectrum of the red phase of chlorophyll *b*. In the case of a moderately thick layer individual bands are not discernable at first until, after a few seconds, the terminal absorption area of the more strongly refracted portion that extends far toward the red end up to the yellow is resolved into two strong bands in the green at $\lambda = 530$ and 495 $\mu\mu$. A band first appears very gradually in the red at $\lambda = 650$, which in the course of a few minutes becomes very dark and wide; the bands in the green continually recede and a new band becomes visible in the yellow at 575. Finally, after about 10 minutes, the pure green color of the alkaline solution, with the characteristic spectrum of component *b*, has returned.

The yellow phase of chlorophyll *a* shows a similar course in the spectroscope except that the individual steps are more difficult to observe spectroscopically on account of the short period of only a few seconds that elapses before the return of the green color.

The analyses of preparations of both components dried under high vacuum are interpreted by means of formulae that are derived especially from the composition of the simpler and therefore analytically decisive chlorophyllides and pheophorbides; namely:

for chlorophyll *a*: $C_{55}H_{72}O_5N_4Mg + \frac{1}{2} H_2O$ (half hydrate),

for chlorophyll *b*: $C_{55}H_{70}O_6N_4Mg$.

The composition, not only of component *a* but of a large number of chlorophyllides and alphyl pheophorbides, is expressed by formulae with 5½ and 6½ atoms of oxygen. These formulae are not to be doubled; such compounds are rather to be considered as half hydrates and their molecular weights agree with the simple formulae.

By the cryoscopic method in veratrol solution ethyl chlorophyllide (with bound ether), for example, gives the molecular weight 738 instead of 693; by the ebullioscopic method in chloroform, ethyl pheophorbide gives 639 instead of 629 and pheophytin 839 instead of 880. Analysis of the magnesium-free derivatives, pheophorbides *a* and *b*, also shows that a half molecule of water does not occur and that there is no external anhydride formation.

The Absorption Spectra.[19]

The diagrams in plate VI (Chapter XXV) present the absorption spectra of the methyl compounds; those of the chlorophyll components are reproduced in the photographs of plate VIII.

Although four shades are used in the graphical presentation of plate VI the degree of absorption, as measured, is indicated by the following six signs: — dark, — — rather dark, . . . moderate absorption, . . feeble absorption, . very weak, | faint shadow.

Chlorophyll Component a. The spectrum shows seven sharply separated absorption bands in the visible region and the terminal absorption area (VIII), which are arranged according to their intensity in the following series: VIII, VII, I, VI, II, III, IV, V.

Band IV, in the case of chlorophyll preparations fit for use, is far less intense than the third; a rather strong appearance of band IV indicates initial pheophorbide formation. Single bands show up most strongly in each of the regions, the red, indigo blue and violet; the weakest absorption bands lie in the yellow and the green.

The methyl and ethyl chlorophyllides coincide, as regards the position and the intensity of the bands, with the phytyl preparation but

[19] Paper XVII.

the edges of the bands appear to be somewhat more sharply delineated in the simple chlorophyllides.

Our observations agree essentially with what M. Tswett reports concerning the preparations of chlorophyll components which he had separated on a small scale by chromatographic adsorption analysis, but band VI is lacking in Tswett's description, or rather, it appears only in his solutions of greatest thickness as a shadow before the terminal absorption area.

SOLUTIONS OF 0.0431 G. IN 1 L. ETHER (0.001) MOL. IN 20 L.).

Thickness of the layer, in mm.	2.5	10	40	80
Band I	669 —— 655	675 — 648	680—637 . 625	684—596 . 587 (I and II)
" II	619 | 605	623 . . 603	625——600	
" III	—	585 . 570	586 . . . 564	587—561
" IV	—	—	539 . . 523	541 . . . 521
" V	—	—	504 | 489	504 . 488
" VI	462 | 455	465 . 453	471. 468— (VI, VII and VIII)	473— (VI, VII and VIII)
" VII	439—427 (VII and VIII)	444 — (VII and VIII)		
Terminal absorption (VIII)	. . . 415—			

Chlorophyll Component b. The spectrum consists of nine bands between $\lambda = 700$ and $410\ \mu\mu$ in addition to the terminal absorption region (X) which commences before $\lambda = 400$; the bands stand with respect to their intensity in the following order: VIII, II, IX, X, I, IV, III, VI, V, VII.

The chlorophyll *a* band in the red[20] is divided into two bands in the case of the *b* component; likewise the absorption in the orange; a small and weaker band in the green corresponds here to the absorption band of *a* in the yellow. The absorption in the blue (VI of *a* and VIII of *b*), on the other hand, has become extraordinarily intense and is now the strongest band. The solution shows, in a deep layer two very characteristic transmission bands, one in the red at B, the other in the green onward from the line E.

In Tswett's description of this spectrum the first band in the red, as well as the band in the green, is lacking.

20 A faint shadow that was often formerly observed in the extreme red was absent in the best preparations of component *b*.

SOLUTION OF 0.0431 G. IN 1 L. ETHER (0.001 MOL. IN 20 L.).[21]

Thickness of the layer, in mm.				
Band I	666 . 659	667 . . . 659 . 651	673—625	677—582 . .
" II	648 . . . 638	651—635		
" III	—	615 | 611	615 . 609	574 . . . 559
" IV	—	599 . 585	600 . . . 583	—
" V	—	—	571 . 559	—
" VI	—	—	547 . . 530	549 . . . 530
" VII	—	—	506 | 500	
" VIII	467—446			
" IX	433 . . 424	474—	483—	508 . 489—
Terminal absorption (X)	407—			

A colloidal solution of chlorophyll, similarly to the living leaf, shows on comparison with a true solution all its bands much displaced toward the red (see also Chapter III, section 2a). In observations with a Nernst lamp as the source of light the following values were found for chlorophyll *a*.

0.044 G. IN 1 L. WATER, CONTAINING 1 PER CENT ACETONE (0.001 MOLE IN 20 L.)

Thickness of the layer, in mm.	10	20	40
Band I	692—664	712—658	732—650 . .
" II	—	637 | 615	640 . . . 609
" III	—	595 | 581	598 . . 575
" IV	—	—	554 . 534
" V	—	—	510.490 . . .
" VI	466 . . . 455—	471—	477—
Terminal absorption			

From a 10 mm. layer the median line of the main absorption in the red is calculated as $\lambda = 678$ as compared with $\lambda = 662$ for the first band of the spectrum of an ethereal solution of chlorophyll *a*.

[21] Measurements on preparations made according to our recent method.

VII. THE ACTION OF CHLOROPHYLLASE.[1]

1. Definition.

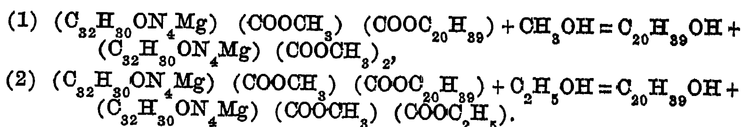
Although the isolation of chlorophyll and its separation into its two components takes place without any chemical reaction; that is, solely by the careful use of solvents, the crystallizable alphyl chlorophyllides and free chlorophyllides, as well as their magnesium-free compounds, were obtained by means of a transformation caused by an esterase of specific action which accompanies the chlorophyll in green leaves and which because of its insolubility is not extractable. This enzyme is designated as chlorophyllase.

The use of the enzyme for preparative purposes, which is discussed in the following chapters, presupposes a knowledge of the method for its detection and of conclusions concerning its distribution as well as of the quantitative determination of its action.

Our attention was called to the enzyme by the observation[2] that, upon the prolonged contact of many leaf extracts with leaf substance, the phytol content of the chlorophyll gradually falls from the normal per cent (33) to almost nothing although no change of the pigment takes place in filtered chlorophyll solutions on standing.

Galeopsis tetrahit, upon rapid extraction of the leaf meal, gave the phytol number 31.3. The same extract, after standing 10 days, gave the phytol number 30.9. The extract, mixed for 3 days with occasional shaking with the extracted Galeopsis meal from which it was obtained, yields pheophorbide with 2.7 per cent phytol.

The reactions that take place under the influence of the enzyme in alcoholic chlorophyll solutions consist of ester transformations, *i.e.*, in the replacement of the phytol radical by a methyl or ethyl group; this is, therefore, an alcoholysis (ethanolysis and methanolysis) of chlorophyll; for example, in the *a*-series:



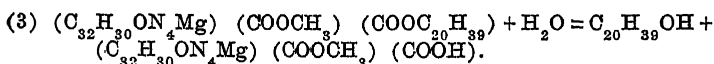
(1) $(C_{32}H_{30}ON_4Mg)\ (COOCH_3)\ (COOC_{20}H_{39}) + CH_3OH = C_{20}H_{39}OH + (C_{32}H_{30}ON_4Mg)\ (COOCH_3)_2$,

(2) $(C_{32}H_{30}ON_4Mg)\ (COOCH_3)\ (COOC_{20}H_{39}) + C_2H_5OH = C_{20}H_{39}OH + (C_{32}H_{30}ON_4Mg)\ (COOCH_3)\ (COOC_2H_5)$.

[1] Papers No. XI, XIII and XIX.

[2] Ann. d. Chem. 378: 4. 1910.

Furthermore, in other solutions containing water, for example, that of ether and especially in dilute acetone, the phytol ester group is hydrolyzed to free carboxyl according to the equation:



$$(3)\ (C_{32}H_{30}ON_4Mg)\ (COOCH_3)\ (COOC_{20}H_{39}) + H_2O = C_{20}H_{39}OH + (C_{32}H_{30}ON_4Mg)\ (COOCH_3)\ (COOH).$$

These reactions are followed quantitatively by means of the phytol number of pheophytin. In ethanolysis, we find throughout the course of the reaction that the ethyl alcohol that has combined is equivalent to the phytol that has been split off. The progress of the reaction can, therefore, be deduced from the increase in amount of the easily volatile alphyl.

A comparison of fresh and steeped leaves shows that the changes of the chlorophyll are really caused by the enzyme. Chlorophyllase is, in fact, especially resistant, as its use in alcohol and acetone of high concentration shows, and it keeps for some time, even on boiling with alcohol, but it is destroyed by a brief boiling of the leaves with water. Transformation of the chlorophyll on contact of its solutions with leaf material remains to be considered.

Five g. of fresh Heracleum leaves were finely ground with some quartz sand and shaken for an hour with 8 cc. of acetone. 95 per cent of the chlorophyll was hydrolyzed.

The experiment was repeated after the leaves had been boiled for 5 minutes. After being pressed between filter papers they still weighed 4 g. They were treated with 1 cc. of water and 8 cc. of acetone as before, and this time no trace of hydrolysis could be detected.

2. Detection of Chlorophyllase.

Plants that are rich in chlorophyllase develop the action of the enzyme excellently when their fresh leaves are placed in aqueous methyl alcohol; for example, 2 g. of Heracleum leaves in 5 cc. of 70 per cent methyl alcohol. The leaf first assumes a deeper green color, then soon becomes lighter colored, especially within a short distance of the leaf veins, and finally yellow. Simultaneously, a portion of the chlorophyll is extracted from the leaves and the solution assumes a light green color. The chlorophyll that is dissolved is wholly methanolyzed according to its solubility ratios; it is insoluble in petroleum ether that contains alcohol. The extract becomes pale greenish yellow in the course of a few hours; it no longer contains any dissolved chloro-

phyll but forms a suspension of beautiful, microscopic crystals of methyl chlorophyllide. Most of the chlorophyll has, however, likewise separated in the leaf in the form of microscopic crystals.

Examination of microscopic sections of whole leaves that have been treated with methyl alcohol gives a more exact picture of the transformation of the chlorophyll.[3] After the leaves are placed in methyl alcohol the chloroplasts assume a darker appearance, the chlorophyll then leaves them and colors the whole cell an intensive green. The palisade cells appear the darkest and the spongy parenchyma is lighter green in consequence of its small chlorophyll content. The

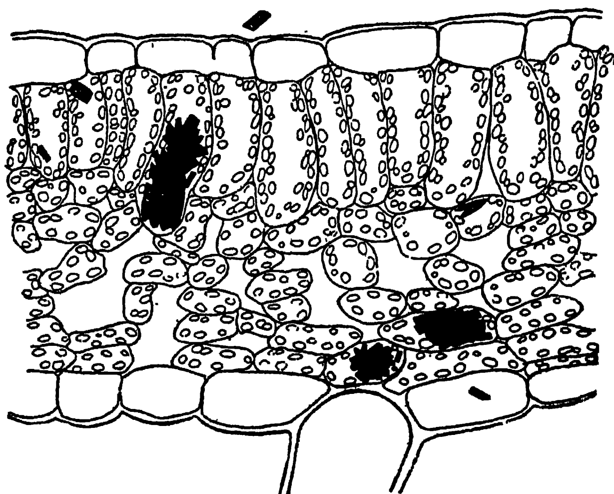


FIG. 6. Section, after methanolysis of a whole Heracleum leaf.

epidermis remains colorless. After the yellowing at the end of the process, if a leaf is held towards the light a host of black spots may be recognized in it with the naked eye. In microscopical sections the chlorophyll is now found to have accumulated in individual cells as rather large crystal clusters which are composed of many, well-formed, rhombic-shaped plates (Fig. 6).

The chlorophyllide is found in this form mostly within the leaf tissue and, at most, but few single crystals lie outside of the tissue.

If, instead of the whole leaves, microscopical sections of fresh leaves

[3] Ann. d. Chem. 387: 336. 1911.

are treated with alcohol, as first described by J. Borodin,[4] much of the chlorophyll leaves the cells during the alcoholysis, and the chlorophyllide finally is largely outside the leaf tissue. It forms beautiful, single crystals under these conditions; the methyl compound forms, as before, rhombic-shaped plates while the ethyl derivative forms very characteristic, triangular and hexagonal plates (Fig. 7). In the

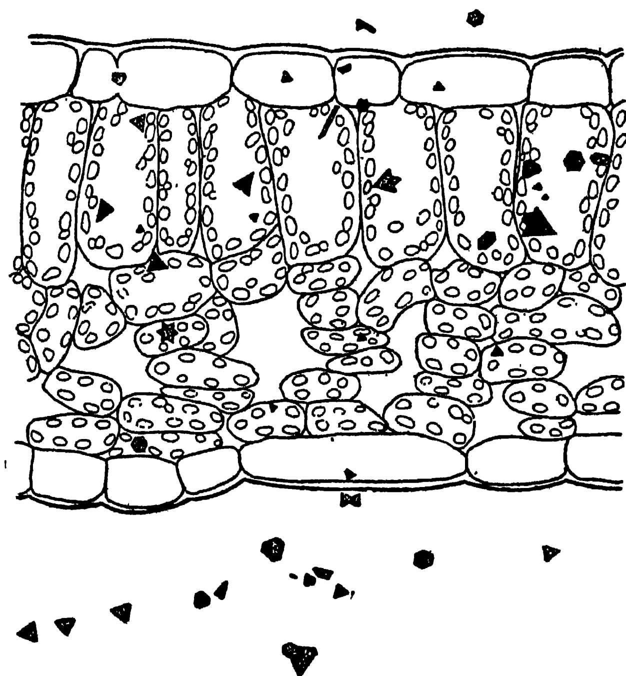


FIG. 7. Ethanolysis of chlorophyll in the section of a Heracleum leaf.

figure, only those crystals that lie within the cells are represented with black surfaces.

If the leaves of plants that are poor in chlorophyllase are treated with alcohol the chlorophyll leaves the chloroplasts similarly, but more slowly, and it precipitates as single, irregularly defined clumps in the leaf tissue.

The solution of the chlorophyll in the different cases and its reprecipitation are explained by the solvent action of the lipoidal chloroplast constituents which mix with the alcohol. If the resulting solution is

[4] Botan. Zeitung 40: 608. 1882.

further diluted with aqueous alcohol it becomes supersaturated with chlorophyll or chlorophyllide. The precipitation then follows quickly if the chlorophyll has become crystalloidal as a result of the alcoholysis.

Small quantities of dried leaf meal may also be tested for their chlorophyllase content by bringing about the formation of the crystallized chlorophyllide by mixing 0.5 g. of the meal with 2 cc. of 85 per cent methyl alcohol.

A qualitative demonstration of the presence of chlorophyllase and an estimation of its effects can be best based upon the more strongly basic properties of the methyl, ethyl and free pheophorbides that are formed upon acidification and which are completely extracted from an ethereal solution even by 22 per cent HCl although pheophytin is, on the other hand, extracted well only by 29 per cent and stronger hydrochloric acid and in traces only by 25 per cent acid: we call this experiment the "Basicity test."

We filter off 1 cc. of the extract that is to be tested and wash the extracted meal thoroughly in order not to lose any of the difficultly soluble chlorophyllides. After the pigment has been transferred into ether and the alcohol or acetone washed out we shake gently with 22 per cent hydrochloric acid. If the acid takes up any pigment, phytol has been split off. If the ether, upon repeated extractions with hydrochloric acid, becomes pure yellow and completely loses its red fluorescence, the enzymatic transformation has been quantitative.

The insolubility of the simple chlorophyllides in petroleum ether, and even in petroleum ether that contains alcohol, also makes possible the detection of the enzyme and a determination of its action. In order to precipitate the derivatives that are insoluble in petroleum ether, it is only necessary to attempt, after filtration from the plant meal, a transfer of the pigment from the extract into petroleum ether by the slow addition of water. With altered chlorophyll the petroleum ether takes up all the green; in the case of a quantitative chlorophyllase action the petroleum ether is yellow. The chlorophyllides that are insoluble in the aqueous and petroleum ether layers are collected upon talc. The portions remaining in the petroleum ether may then in an alcoholic, alkaline solution be compared colorimetrically with the talc portion.

3. Distribution of the Enzyme.

Chlorophyllase is distributed among all the classes of plants that were investigated by us. Its presence has been demonstrated particu-

larly in very many dicotyledonous families, as well as in monocotyledons (Avena sativa), gymnosperms (Taxus baccata), Equisetales (Equisetum arvense), Filicales (Aspidium), Pheophyceae (Fucus) and Chlorophyceae (Ulva lactuca).

But there are only a few plants that are, on account of their general distribution, their abundant occurrence and their large chlorophyllase content, especially adapted at all times of the year as material for the preparative use of the enzyme; namely:

Heracleum spondylium, hogweed or cow parsnip;
Galeopsis tetrahit, hemp nettle;
Stachys silvatica, hedge nettle.

Lamium maculatum, Datura stramonium and Melittis melissophyllum are also well adapted for this purpose.

Many plants, such as Aesculus hippocastanum, contain an abundance of chlorophyllase in the fresh condition but quickly lose their strength on drying.

A further group of plants contains so little enzyme that no considerable change of chlorophyll occurs even upon slow extraction or in experiments that require a considerable period of contact of the extracts with the leaves. To this group belong:

Grass,
Plane tree,
Stinging nettle.

4. Application of the Enzyme.

Since the enzyme occurs alongside its substratum (chlorophyll) in the green parts of the plant, the freshest possible, undried leaf material was brought in contact with the extract (which was being made or had just been made) in many cases where the chlorophyllase was used to bring about alcoholysis and hydrolysis.

Upon this rests the method for obtaining methyl chlorophyllide and free chlorophyllide, Chapter IX, 3 and 4.

It was possible to work up the leaves, mostly in daily charges of 20–30 kg., in the laboratory in the fresh state a few hours, or at most a day, after their collection—during this time they lay spread out in cool rooms.

The working up of undried leaves requires, in consequence of their water content, an extraordinary consumption of solvents and capacious

apparatus. Besides, this work is dependent upon the proximity of the habitat of suitable plants and upon the time of the year. Consequently, the use of the meal of dried leaves is often preferable.

The preparation of ethyl and methyl chlorophyllides in Chapter IX, 1 and 2, and the partial synthesis of chlorophyll from chlorophyllide and phytol, Chapter VIII, are examples of this.

Drying should take place quickly, in 1–2 days, when the leaves are spread out in thin layers at temperatures not higher than 40° and with avoidance of the sunlight. We dried the leaves mostly over the steam boiler of the laboratory or had them dried by the herb collectors in drying sheds.

The enzymatic activity of the most suitable plants suffers only upon prolonged storage of the dried material; yet crystalline chlorophyll was quite successfully obtained from Galeopsis or Heracleum that has been kept months or even years.

Three-year-old leaves of Galeopsis or Heracleum were still active and, in fact, the half transformation period in ethanolysis was only four times that with new comparison material. Ethanolysis of 44 per cent of the chlorophyll in the extract (80 per cent alcohol) of the old leaves occurred in 10 hours at 25° when it was treated with 1/10 of the leaf meal from which it had been extracted.

If the enzyme was not to be used for preparative purposes but for kinetic experiments (for example, in sections 6, 7 and 8 of this chapter) the meal of leaves dried at room temperature was used as chlorophyllase after a preliminary quick, and then thorough, extraction of the chlorophyll with 96 per cent alcohol and, in fact, it was used while still moist with alcohol as soon as possible after the extraction and at times the leaf meal was used directly without preliminary treatment.

The amount of the enzyme is then expressed as the fraction of the plant meal that corresponds to the chlorophyll content of the extract used in the experiment. For example, 1 kg. of Galeopsis furnished an extract with 4.14 g. of chlorophyll corresponding to 3 g. of ethyl chlorophyllide, while the total chlorophyll content of the meal amounted to 6.9 g.; 60 g. are then designated as 1/10-enzyme; namely 1/10 of that material which contains 4.14 g. of chlorophyll.

5. Determination of the Hydrolysis by Separation with Alkali.

The progress of the hydrolysis may be determined by a simple colorimetric method that depends upon the acid nature of the free chlorophyllide.

After the reaction, samples of the pigment; for example, 0.01 g., or in experiments on a small scale, the whole amount, are transferred to ether (100 cc.) and the acid portion is extracted with 0.02 *N* KOH to which a few cc. of methyl alcohol has been added; about 3 times with 30–50 cc. If much chlorophyllide forms, the potassium salt often separates in the form of an emulsion at the boundary surface between the lye and the ether; it is brought into solution with more dilute lye. The united alkaline extracts are made up to a volume of 200 cc. with methyl alcohol; by this means the solution is prevented from quickly becoming discolored. The chlorophyll remaining in the ether is then saponified by the addition of 5 cc. of methyl alcoholic potash and, after the return of the green color, it is similarly diluted with 100 cc. of water and with methyl alcohol to 200 cc. The two solutions are compared in the colorimeter; the error in this amounts to $\pm$ 2–3 per cent.

In two experiments with Heracleum under the same conditions we found thus, in good agreement, 70 and 71 per cent of hydrolyzed chlorophyll.

The following conditions are suited to the investigation of the enzymatic action of plant material by this method.

The hydrolysis is carried out in 66 per cent (by volume) acetone. The meal of leaves that have been dried to constant weight (1 g.) is first shaken for 5 minutes with 4 cc. of pure acetone; this length of time is sufficient for good extraction and the extract contains only unaltered chlorophyll. Two cc. of water are then added while shaking, whereupon the meal swells up and becomes darker colored. This mass is stirred in a thermostat at 20°, usually for one hour. The chlorophyll solution is then filtered off upon a "Nutsch" and washed thoroughly with 66 per cent acetone.

In the examination of fresh leaves the water content is determined and enough acetone is used to make the solution 66 per cent; for example, 5 g. of Heracleum leaves gave 1 g. of dry substance, consequently they were ground with quartz sand and treated with 8 cc. of acetone. The solutions are always more dilute than when working with dried plants.

First example with dried leaves of Heracleum.—The plant was collected in October and was immediately dried for two days in a desiccator. After 7.5 minutes 30 per cent, and after 15 minutes 56 per cent, of the chlorophyll was hydrolyzed.

Second example with fresh (undried) leaves of Heracleum.—The hydrolysis proceeded more slowly, corresponding to the smaller chlorophyll concentration. Consequently, in the case of Heracleum the enzyme activity does not suffer as a result of quick drying. After 7.5 minutes 20 per cent was hydrolyzed and after 30 minutes 50 per cent.

6. Determination of the Alcoholysis by Means of the Phytol Number and the Silver Iodide Number.

The progress of the alcoholysis can be ascertained through the difficultly soluble chlorophyll derivative that separates from the alcoholic solution upon the action of oxalic acid by the quantitative determination of its phytol content according to the method described in Chapter XVII, as well as by the quantitative splitting off of its methyl and ethyl groups with iodine water by Zeisel's method.

(a) *Phytol Number.* Upon acidification of the alcoholic solution after the enzymatic action the magnesium-free derivative precipitates incompletely. But it happens that pheophytin and pheophorbide separate in approximately the same proportion as that in which they are contained in the solution.

For example, a solution of chlorophyll from stinging nettle gave, after its ethanolysis, upon acidification and two days' standing, 0.6 g. of pheophytin with the phytol number 15.3; the mother-liquor, on evaporation to half its volume and after standing two days, produced an additional 0.5 g. with 15.1 per cent of phytol. An extract of Galeopsis, after the enzyme reaction, produced, on standing, 0.75 g. of precipitated pheophytin with a phytol number of 2.4; the mother-liquor, after concentration to half its volume, produced 0.45 g. with 3.8 per cent of impure phytol.

We can, therefore, often be content with analyzing the portion of the pheophytin that separates from the acidified chlorophyll solution when this stands two days.

From the phytol number (Z_u) of the precipitated mixture of pheophytin and ethyl pheophorbide and the phytol number, (Z_a), of the magnesium-free derivative of the extract or chlorophyll preparation that was used, the fraction of the chlorophyll that has been altered, that is, the transformation number u_I, is derived according to the following equation.[5] The degree of transformation, however, does not increase proportionally to the decrease of the phytol number.

[5] Ann. d. Chem. 378: 32. 1910.

$$u_I = \left(1 - \frac{Z_u}{Z_a(1 + 0.01180)\ (Z_a - Z_u)}\right) \times 100. \qquad \text{(I)}$$

The formula, $\frac{a}{a-u} = \frac{Z_a(1+0.01180)\ (Z_a - Z_u)}{Z_u} \times 100$, serves for the application of the equation to the monomolecular reaction:

$$k = \frac{1}{t} \times \ln \frac{a}{a-u}$$

(b) *Silver Iodide Number.* Chlorophyll and pheophytin contain a methoxyl and, in alcoholic solution, take up an ethoxyl group through the action of chlorophyllase. Partially transformed preparations contain methoxyl and ethoxyl in varying proportions. Consequently, it is inexpedient to report the results as methoxyl or ethoxyl. Instead of this, we introduce the concept of the silver iodide number, the quotient:

$$\frac{\text{Ag I found}}{\text{material used}} \times 100.$$

The theoretical silver iodide number of ethyl pheophorbide and, in fact, of the component *a*, the chief constituent of a mixture of *a* and *b*, and, without any considerable error, that of the mixture is:

$$\frac{469.6}{629.35} \times 100 = 74.6.$$

The theoretical silver iodide number of pheophytin is:

$$\frac{234.8}{879.6} \times 100 = 26.7.$$

The silver iodide number of an alcoholyzed preparation, which shall be designated as J_u, lies between these two values.

The transformation number u_{II} is obtained from the silver iodide numbers by means of the following formula,[6] in which D_a signifies the difference between the silver iodide number of ethyl pheophorbide and that (J_a) of the pheophytin of the chlorophyll that was used:

$$u_{II} = \frac{1}{1 + \frac{74.6 - J_u}{(1 + 0.00830\ D_a)(J_u - 26.7)}} \times 100. \qquad \text{(II)}$$

Instead of ascertaining the extent of the transformation from the silver iodide numbers before and after the reaction, the initial material

[6] Ann. d. Chem. 378: 34. 1910.

may be designated simply by its phytol number (Z_a) and the extent of the transformation (u) then computed by aid of the silver iodide number (J_u) in the following manner:

$$u_{III} = \frac{1}{1 + \frac{74.6 - J_u}{(1 + 0.01180 \cdot Z_a)(J_u - 26.8)}} \times 100. \qquad \text{(III)}$$

The agreement in the degrees of transformation, u_I and u_{II}, as derived from the phytol and silver iodide numbers, teaches us, as the following examples show, that the ethoxyl entering the molecule is exactly equivalent to the phytol leaving it, and at the same time it proves the usefulness of the two methods.

Alcoholysis of pheophytin.

Values for the preparation used: $Z_a = 30.9$; $J_a = 29.9$.

Values for the altered preparation: $Z_u = 11.0$; $J_u = 58.3$; $u_I = 71.2$; $u_{II} = 72.7$; $u_{III} = 72.6$.

Values for the same preparation still further alcoholyzed: $Z_u = 2.7$, $J_u = 70.2$, $u_I = 93.4$, $u_{II} = 93.4$, $u_{III} = 93.4$.

These two methods of quantitative determination cannot be carried out with small samples and they require considerable time. They are, therefore, not so well adapted to the control of experiments on a preparative scale as the tests given in sections 2 and 5.

7. Dynamics of the Enzymatic Reaction.

Velocity measurements under different conditions, which permit the recognition of the influence of solvents, especially of water, temperature, age of the enzyme preparations and other conditions, serve for the determination of suitable experimental conditions for using chlorophyllase. The conditions for measuring the velocity of reaction are especially unfavorable here because of the nature of the enzyme which, being used in the form of extracted plant meal, is extraordinarily diluted with other materials, and, on the other hand, because of the complex, and for the most part unknown, composition of the leaf extract that contains the substratum.

The chlorophyllase reaction does not take place in a homogeneous system, but the diffusion conditions could be such that the heterogeneous system would behave as a homogeneous one. It would then be possible for the reaction between chlorophyll and the alcohols (whose change in concentration need not be considered) or water to proceed

as a monomolecular reaction. Experiments, however, have shown that the reaction constant

$$k = \frac{1}{t} \times \ln \frac{a}{a - u}$$

varies considerably; namely, that it falls considerably as the duration of the reaction increases.

The explanation for this is to be sought, on the one hand, in the fact that the enzyme is weakened or destroyed during the reaction, for its activity is lessened with repeated use. Furthermore, it is more likely that a co-enzyme or activator, whose influence is apparent, be-

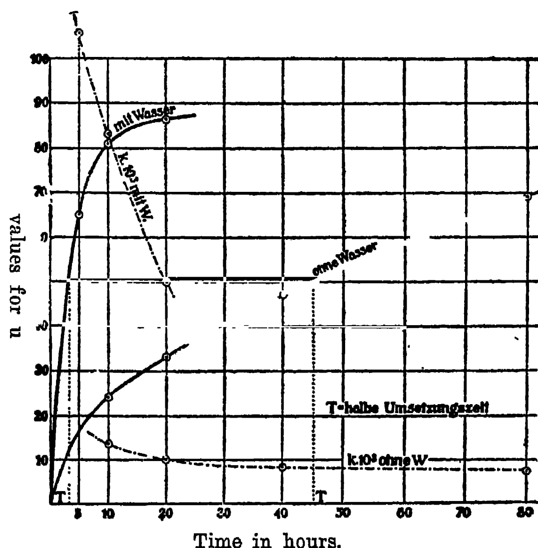


FIGURE 8.

comes weaker during the course of the reaction. Finally, it is probable that the conditions of diffusion, considering the peculiar nature of the enzyme-containing material, exert a disturbing influence upon the velocity under our experimental conditions.

Examples

The extracts should contain as much undamaged chlorophyll as possible; they are consequently prepared quickly by the "Nutsch" method. The concentration of the solvent is given in per cent by volume. The moisture contained in the plant meal is considered in the

determination of the water content of the extract. The experiments were carried out while shaking at constant temperature.

1. *Hydrolysis.*

Heracleum, desiccator dried, with all its enzyme in 66 per cent acetone at 20° C. Experimental conditions and determination as in section 5.

Time in minutes	u	$k \times 10^3$
15	45	7.52
30	75	8.68
60	91	7.55
120	98	6.17
240	99	—

2. *Ethanolysis* (Fig. 8).

Heracleum (harvested during the first part of May) in 92 per cent alcohol at 25° with 1/10-enzyme; 500 cc. of extract containing 1.8 g. of chlorophyll mixed with 27.1 g. of leaf meal.

$Z_a = 31.5$

Time in hours	Z_u	u	$k \times 10^3$
10	25.6	24.0	27.5
20	23.2	33.1	20.0
40	19.1	47.1	15.9
80	12.0	69.0	14.7

Influence of the addition of water (the same figure):

Heracleum (harvested the latter part of May) in 80 per cent alcohol at 25° with 1/10-enzyme, as above.

$Z_a = 30.6$

Time in hours	Z_u	u	$k \times 10^3$
5	12.9	65.1	210.7
10	7.4	81.0	166.1
20	5.4	86.4	99.8

The velocity, therefore, is much greater. The improvement shown by the addition of water appears even more certain in a parallel experiment with a similar extract in 92 and in 80 per cent alcohol.

Heracleum with 1/10-enzyme; 25°, 10 hours.

$Z_a = 30.6$

In 92 per cent alcohol: $Z_u = 19.5$; $u = 43.7$; $k \times 10^3 = 57.4$.

In 80 per cent alcohol: $Z_u = 9.4$; $u = 75.4$; $k \times 10^3 = 140.3$.

The half transformation periods may be evaluated sufficiently accurately by means of the curves of Figure 8; they are 44[7] and 4 hours.

Even with such a significant, and with a still greater, water content of the alcoholic solution there is only a trace of hydrolysis accompanying the alcoholysis.

Influence of Temperature:

The optimum temperature for ethanolysis is lower than that found for the lipases of castor oil seed and pancreas; namely, at about 20°.

Heracleum in 80 per cent alcohol with 1/10-enzyme; time 4 hours

$Z_a = 31.9$

20°	$Z_u = 23.4$	$u = 33.3$
25°	$= 25.6$	$= 25.3$
35°	$= 26.5$	$= 21.9$

Heracleum in 80 per cent alcohol with 1/10-enzyme; time 5 hours

$Z_a = 31.7$

15°	$Z_u = 21.0$	$u = 41.2$
20°	$= 19.1$	$= 47.6$
25°	$= 21.6$	$= 39.1$

Diminishing action of the enzyme:

We first ascertained the velocity for a preparation of enzyme and extract in 3 experiments of 2.5, 5 and 10 hours. The enzyme from each experiment was allowed to act again for 2.5 hours upon the original chlorophyll solution. It was seen that the strength of the enzyme remained unaltered in the 2.5 hours of the first experiment. The enzyme that had been active for 5 and for 10 hours proved to be less active and, in fact, to such a degree that the difference between the constants of these three repetition tests explains, in part, the decrease of our reaction constants as due to the weakening of the enzyme during the course of the experiment.

Heracleum in 80 per cent alcohol at 25°: 500 cc. of extract, containing 1.95 g. of chlorophyll, mixed with 30 g. of leaf meal (1/10-enzyme).

[7] *Trans.* From Figure 8 it is evident that this should be 44 and not 14, as given in the German edition.

$Z_a = 31.9$

Experiment	Time in hours	Z_u	u	$k \times 10^3$
I	2.5	26.0	23.8	108.7
II	5	23.0	34.8	85.4
III	10	17.0	54.7	79.1

Action repeated for 2.5 hours:

	Z_u	u	$k \times 10^3$
Enzyme from experiment I	25.9	24.2	110.7
" " " II	27.2	19.2	85.4
" " " III	28.1	15.7	68.3

Chlorophyllase of One Plant with the Chlorophyll Solution of Another.

Plants poor in chlorophyllase give extracts that do not react, as regards alcoholysis and hydrolysis, as well as extracts from plants containing the enzyme. The same plants give extracted leaf meals that do not bring about, with solutions that are most suitable for the reaction, as large a transformation as good enzyme material.

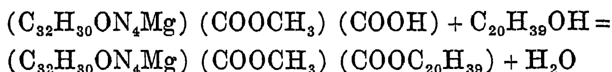
Time: 60 Minutes.

Stinging nettle meal with stinging nettle extract: 10% hydrolysis,
" " " " Heracleum " : 23% "
Heracleum meal with stinging nettle extract: 62% "
" " " Heracleum " : 91% "

VIII. APPLICATION OF THE ENZYME TO PARTIAL CHLOROPHYLL SYNTHESIS.[1]

Just as ester and glyceride synthesis was successfully accomplished with pancreas lipase[2] so was also the inverse of alcoholysis, that is, the transformation of ethyl chlorophyllide into the phytol ester, attained with chlorophyllase. Since the enzyme requires the presence of water, in which phytol is insoluble, the conditions are not favorable to the reaction; the yield was, in consequence, small.

The rather important case of the esterification of the free carboxylic acid chlorophyllide with phytol, according to the equation:



$$(C_{32}H_{30}ON_4Mg)\ (COOCH_3)\ (COOH) + C_{20}H_{39}OH =$$
$$(C_{32}H_{30}ON_4Mg)\ (COOCH_3)\ (COOC_{20}H_{39}) + H_2O$$

could be realized in a satisfactory manner in a very simply arranged experiment, even before it was possible to isolate the easily decomposable chlorophyllide as such.

The meal of Galeopsis leaves is digested several days with moist ether. The phytol ester is split hydrolytically; the phytol number falls from an initial value of 32.8 to a few per cent. Phytol is now added to the ethereal solution with which the leaf meal has been mixed. A larger part of the chlorophyll, namely, one-third to three-fourths, is then reformed in the course of several days; this is indicated by the rise in the phytol number.

Hydrolysis.

1 kg. of the meal of Galeopsis leaves (harvested in August) was mixed with 20 g. of precipitated calcium carbonate and 2.2 l. of alcohol-free, moist ether and macerated for 3.5 days with occasional shaking. Even after a half hour a filtered sample gave a somewhat too low phytol number, namely, 30.4.

[1] See paper XIII.

[2] J. H. Kastle and A. S. Loewenhardt. Amer. Chem. Journ. 24: 491. 1900; H. Pottevin, Compt. rend. 136: 1152. 1903; 138: 378. 1904; Bull. soc. chim. 35: 693. 1906; M. Bodenstein and W. Dietz, Zeitschr. f. Elektroch. 12: 605. 1906 and W. Dietz, Zeitschr. f. Physiol. Chem. 52: 279. 1907.

Our calculation of the transformation is not based upon this initial phytol number but upon the theoretical value for pheophytin *a*, 33.7.

The chlorophyll shows altered solubility relations after several days action. If a sample is transferred from the ether into an alcoholic solution and an attempt is made to transfer the chlorophyll into petroleum ether the pigment is almost completely precipitated.

In order to determine its phytol number, the chlorophyllide that was formed was isolated in the form of its magnesium-free derivative in the following manner. The meal was shaken well with its ether extract and half of it was removed in order to investigate the action of the phytol in the remainder. The filtered and washed ethereal solution was concentrated to 250 cc., mixed with 500 cc. of but 85 per cent alcohol (the pheophorbides would be too soluble in alcohol of higher concentration) and concentrated in vacuum at 25° to 300–400 cc. The alcoholic chlorophyll solution was shaken with 50 g. of calcium carbonate for the purpose of purification, whereupon a large quantity of yellow and colorless materials precipitated and the solution cleared to a pure green. The alcoholic solution was diluted to 80 per cent and the clarification with calcium carbonate repeated. It was finally acidified with oxalic acid and the magnesium-free derivative was reprecipitated from chloroform with 85 per cent alcohol.

The extent of hydrolysis and later that of esterification is calculated from the phytol number just as was the course of alcoholysis previously. The formula is simplified since Z_a may here be placed equal to the theoretical phytol number Z_0.

$$\frac{M_1}{M_2} = 1.4629,$$

M_1 and M_2 signify the molecular weights of pheophytin *a* (879.6) and pheophorbide *a* (601.3).

$$u = \frac{1.4629\left(1 - \frac{Z_u}{Z_0}\right)}{\frac{Z_u}{Z_0} + 1.4629\left(1 - \frac{Z_u}{Z_0}\right)} \times 100.$$

The silver iodide numbers of the hydrolyzed preparations always turn out to be somewhat too high. Either a little ethyl alcohol has entered during the otherwise careful and rapid isolation or the ether, under the influence of the chlorophyllase, has had a slight ethylizing action.

Esterification with Phytol.

After completion of the hydrolysis the remaining half of the Galeopsis meal (0.5 kg.) and Galeopsis extract was mixed with about 50 g. of phytol; that is, about 50 moles referred to the chlorophyll content of the leaves. The action was allowed to take place for three days. The ethereal solution was then filtered from the plant meal and a concentrated alcoholic solution was prepared from it just as in the case of hydrolysis and this was clarified. We then acidified with oxalic acid and obtained in a few hours the first precipitation of pheophytin. The mother-liquor, as a consequence of its phytol content, still retained a considerable quantity of dissolved pheophytin. In order to separate the remainder, the substance was transferred into ether and, after washing out the alcohol, was completely evaporated in a vacuum. The residual solution of pheophytin in phytol was diluted with 2 l. of petroleum ether and then allowed to stand in the cold. The pheophytin separated rather completely and the petroleum ether mother-liquor contained hardly anything more than yellow pigment. The two fractions of pheophytin, or more precisely of the mixture of pheophorbide and pheophytin, were combined and reprecipitated, first from a concentrated chloroform solution with 85 per cent alcohol, then a second time from chloroform with petroleum ether. In this way the product was obtained in a pure form; colorless accompanying substances and carotin remained in the chloroform-petroleum ether mother-liquor.

Example. (a) Hydrolysis.

The isolated solution contained, after clarification, 1.8 g. of chlorophyll, calculated as phytol-containing chlorophyll. The yield of pheophorbide amounted to 1.05 g.; that is, 85 per cent of theory.

Phytol number $Z_u = 3.0$,
Silver iodide number $J_u = 43.3$.

The theoretical value for pheophorbide *a* is 39.0.

The silver iodide number shows that hydrolysis, and not alcoholysis, has taken place.

The extent of the transformation, u_1, estimated only from the phytol number, = 94.

(b) Esterification.

Yield of chlorophyll in the solution, 2.0 g. and 1.45 g. of twice reprecipitated pheophytin; that is, 85 per cent of theory.

$$Z_u = 17.2$$
$$J_u = 30.3$$
$$u_2 = 35.$$

In this experiment, therefore, 38 per cent, in another, 73 per cent, and in a third 29 per cent of the free carboxylic acids that were formed combined with phytol.

The chlorophyll remained intact in its phytochromin complex during the hydrolysis and synthesis. The magnesium-free derivative of the reaction product yielded, in fact, on saponification a normal mixture of phytochlorin *e* and phytorhodin *g* and no other basic cleavage products.

When the action of ethyl alcohol was tested on portions of the ethereal solution of hydrolyzed chlorophyll parallel to the ester formation by means of phytol, esterification with 25 moles of the ethyl alcohol gave approximately the same result as double that number of phytol molecules.

This explains why, in the extraction of Galeopsis meal with ordinary, not wholly alcohol-free ether, the Borodin crystals are obtained in small amounts.[3]

Formation of Chlorophyll a from Phytol and Free Chlorophyllide a.[4]

After we had learned to know the unstable (and consequently difficult to isolate) free chlorophyllides, we succeeded in a very simple manner in forming natural chlorophyll from the two isolated constituents, its alcohol and carboxylic acid.

Chlorophyllide *a* and phytol were brought into solution and mixed with a little meal from leaves that were rich in chlorophyllase. The synthesis of the neutral ester could be followed by the decrease of the acid constituent, which we extracted from the ethereal solution with 0.02 *N* KOH.

We dissolved 0.2 g. of chlorophyllide *a* in 4 cc. of acetone because it was difficultly soluble in phytol; the beautiful, greenish blue solution was introduced into 16 cc. of phytol and mixed with 10 g. of air-dried meal of Heracleum leaves (that is, one-fourth that corresponding to

[3] Ann. d. Chem. 358: 275. 1907 and 378: 59. 1910.
[4] Unpublished.

the chlorophyllide used). The viscous mass was stirred often and 2 g. samples were weighed out from it from time to time and extracted and washed upon a small "Nutsch" with much ether (100 cc.). The extract was then repeatedly shaken gently (with the addition of a little alcohol in order to avoid emulsions) with 50–100 cc. of 0.02 *N*–KOH till the aqueous layer remained colorless. The combined extracts were diluted with alcohol to 500 cc. and compared colorimetrically with a solution that had been prepared in the same manner at the beginning of the enzyme reaction. Since the aqueous, alkaline, chlorophyllide solution easily becomes discolored on standing, several such initial tests are taken from the reaction mixture and stored in the dark, in the form of their dilute ethereal solutions, in order that a comparison solution may be prepared at the same time with the test sample.

Under the above-stated experimental conditions with dried leaf meal, no distinct decline of the acid constituent was perceptible even after 1 and 2 hours. After 24 hours 20 per cent of the chlorophyllide that was used had been synthesized.

Chlorophyllase acted only very slowly in the almost anhydrous medium but, on the contrary, after vigorous agitation with 0.5 cc. of water, 60 per cent of the carboxylic acid that was originally present was esterified in an additional day. The enzyme reaction had already almost reached equilibrium at this point, for in the course of another day the yield of chlorophyll increased to only about 65 per cent and there was no further increase during the two days that followed.

The concentrated solution of chlorophyllide *a* in phytol does not form any ester when no enzyme is added, even on standing for days.

The ethereal solution of synthesized chlorophyll showed the characteristics of the natural pigment; it was indifferent toward dilute aqueous alkalies; acted like its magnesium-free derivative toward 23 per cent hydrochloric acid (basicity test) and showed a pure yellow phase.

IX. APPLICATIONS OF CHLOROPHYLLASE FOR MAKING PREPARATIONS: THE CHLOROPHYLLIDES.

In our first investigations[1] the phytol content of chlorophyll and pheophytin was found to be irregular, either normal or else too low, and Willstätter and Benz have described an empirical procedure for the production of phytol-free, "crystallized" chlorophyll without having any knowledge of the exact circumstances that govern its formation. In chlorophyllase we found the key to the relations between phytol-containing and phytol-free chlorophyll; an investigation of the enzyme reaction was then necessary before chlorophyllase could be usefully employed for preparative purposes.

The isolation of natural chlorophyll has been until recently, on account of its great solubility and instability and the absence of acid and basic properties, so difficult that for its analytical investigation and for the first steps of its systematic decomposition the pigment was preferably isolated and used in the form of its difficultly soluble and excellently crystallizing, simpler chlorophyllides. Besides, the differences in composition between the *a* and *b* series appear more pronounced in those phytol-free compounds by means of which the resolution of the natural mixture into its components was first carried out.

Even after the new method which is described in Chapter V has made it possible to obtain chlorophyll in a pure condition and in any desired quantity, the so-called crystallized chlorophylls, the most beautiful substances of the chlorophyll group, retain their importance as initial materials of reliable purity, especially for the first transformations of the pigment.

1. Ethyl Chlorophyllide ("Crystallized Chlorophyll").

J. Borodin[2] in 1881 discovered crystallized chlorophyll in microscopical leaf sections when he treated them with ethyl alcohol and

[1] Papers VII and X.

[2] Bot. Zeitung 40: 608. 1882.

allowed them to dry under the cover-glass, and described in a splendid manner the form of the crystals. He raised the question whether the remarkable forms that were presented were the natural pigment or a compound of it with a still unknown substance.

N. A. Monteverde,[3] who considered the Borodin crystals to be natural chlorophyll and the amorphous form a decomposition product, prepared (1893) the crystallized product in order to describe its absorption spectrum and gave the following procedure for doing so:

"Fresh leaves were freed from their larger veins, cut up finely with shears, then washed with alcohol and treated with cold, 95 per cent alcohol. After one hour the alcoholic solution was filtered and evaporated in the open air. The crystals that separated were freed from all foreign admixtures and pigments by means of distilled water and benzine."

This inspiring work of Borodin and of Monteverde was not appreciated anywhere in the literature except by a Russian botanist, M. Tswett; probably because no chemical investigation of the chlorophyll crystals was made and because later authors did not succeed in isolating the chlorophyll in the form described.

Willstätter and Benz (1907), by following the directions of Borodin and Monteverde, again obtained crystallized chlorophyll and succeeded in devising a method for the isolation of the crystals in large quantities.

They used dried leaves instead of fresh ones, transferred the pigment from the alcoholic extract into ether, and obtained the crystals by a purification of this solution.

A. Gautier[4] later on mentioned that he had isolated[5] crystallized chlorophyll even before the observations of Borodin and Monteverde and that he had recognized, upon the basis of the phosphate content of the ash of his preparations, the essential rôle of phosphorus in chlorophyll.

Upon comparison of the published composition of Gautier's product with that of the alkylchlorophyllides, it is seen, however, that Gautier's chlorophyll preparation which possessed acid properties and formed soluble alkali salts, was a decomposition product.

[3] Acta Horti Petropolitani No. 9. 13: 123. 1893.

[4] Bull. soc. chim (4) 5: 319. 1909.

[5] Compt. rend. 89: 861. 1879.

Gautier's chlorophyll		Ethyl chlorophyllide (crystallized chlorophyll according to Borodin)	
C	73.97		68.57
H	9.80		5.97
N	4.15		8.80
Ash, phosphates	1.75	Ash (=MgO)	5.92

The Procedure.

Ten kg. of meal from Galeopsis tetrahit are extracted for 2–3 days with 20 l. of 96 per cent alcohol, then filtered off under suction and washed. An ethereal solution is prepared from the extract by mixing it with 20–25 l. of ether and then adding the required amount of water (60 l. with 0.25 l. of a saturated sodium chloride solution) for the removal of the greater portion of the alcohol. It still contains disagreeable impurities which are difficult to separate, an indifferent, mucilaginous material which hinders by the formation of emulsions any further removal of the alcohol by washing. In order to remove the mucilage, the liquor is shaken repeatedly for hours at a time with clarifying media; best, three to four times with a whole kilogram of talc each time. Even then a portion of the impurity still remains in the ether, but it no longer causes troublesome emulsions when shaken with water and it is almost entirely removed when this is done. The solution is to be shaken five more times with water, using 20 l. of this each time and keeping the volume of the solution undiminished by replenishing it with ether. The ethereal solution is finally concentrated in the water-bath to 3 l. The greater portion of it then crystallizes out in a few hours upon standing; this is washed upon the filter with ether (to free it from the mother-liquor and the yellow accompanying substances) until the wash liquor runs off with pure light green color. Upon further concentration, the filtrate furnishes more crystallizations of slightly less purity.

The yield amounted to 17 g., of which 13 were isolated as the first crystallization. When working up smaller quantities this yield is easily surpassed.

An improvement on this method by Willstätter and Utzinger[6] makes practical application of their knowledge regarding the enzymatic process and achieves complete alcoholysis. It also simplifies the

[6] Ann. d. Chem. 382: 142. 1911.

cumbersome procedure involved in the purification with talc. Two points in the directions are considered essential:

1. Action of the leaf meal upon the extract for a sufficient length of time.

2. The addition of water in order to promote enzyme action.

2 kg. of the ground leaves of a plant that is rich in chlorophyllase are mixed with 4 l. of spirits. Several hours later 400 cc. of water are dropped in while the vessel's contents are rotated and the alcoholysis is then allowed to proceed with frequent shakings. Its progress is controlled by the test-tube method of transferring the chlorophyll into petroleum ether. The pigment precipitates quantitatively from this when the cleavage of the phytol has been completed.

This may happen the same day and will certainly occur by the next morning.

The pigment is transferred from the alcoholic solution, which has been filtered from the leaf meal, into ether and the greater portion of the alcohol is washed out. In doing this, vigorous shaking is not permissible, else emulsions would form. The ethereal solution is then dried with sodium sulphate and evaporated till it begins to become viscous. Without considering any separation that may have occurred a thin paste is formed by the addition of talc; this is shaken a short time and allowed to stand a half to a whole day. It is then subjected to strong suction upon a "Nutsch" and thoroughly washed with ether till this runs off with a pure and very light green color. The talc is interspersed throughout with beautifully formed, triangular and hexagonal, microscopic crystals and does not contain any yellow pigments.

The talc is extracted as quickly as possible (10–15 min.) with not quite absolute alcohol in order to isolate the chlorophyll crystals. Since alcohol does not act upon the pigment in the extract but does so in the pure solution and renders it non-crystallizable as a result of allomerization, the solution that has been obtained is quickly mixed with ether and the alcohol washed out completely. Chlorophyllide, free from yellow and colorless admixtures, crystallizes from the ethereal solution when this is moderately concentrated.

New Method.[7]

In the method of Willstätter and Utzinger enzymatic alcoholysis was complete but isolation was brought about only with difficulty and

[7] Unpublished.

losses. Our modification increases the yield and makes it possible to obtain the two yellow pigments, without trouble and almost completely, as secondary products.

During the enzyme reaction a portion of the chlorophyll crystallizes in the plant meal. Instead of washing with alcohol after the extract has been filtered off, the meal is washed with acetone which, even though it becomes slightly aqueous, will nevertheless quickly and easily dissolve ethyl chlorophyllide. The pigment is no longer transferred from the whole filtrate into ether, but is slowly precipitated in the form of crystals by means of water. These can be washed free from colorless and yellow substances.

The meal of Heracleum, Galeopsis, or Stachys leaves is mixed with only 90 per cent alcohol, 1 kg. with 2 l., and is permitted to stand for 12 hours with occasional shaking.

After a few hours, in the case of a good enzyme content, the "Basicity Test" shows 80 per cent alcoholysis. After 12 hours a test sample, transferred to ether, should give up all its chlorophyll pigment to 22 per cent hydrochloric acid, as ethyl pheophorbides *a* and *b*, so that the ethereal layer becomes pure yellow and non-fluorescent after two extractions with this acid.

At the same time testing with alkali shows that no free chlorophyllide has been formed. The water content of the alcohol does not cause any considerable extent of hydrolysis in addition to the ester alteration. In good experiments, a sample of extract transferred to ether does not color 0.01 *N* KOH when thoroughly shaken with it.

At the conclusion of the enzyme reaction filter upon a suction filter and wash with acetone, using 2.5 l., in half liter lots, by alternately macerating for a short time and then filtering off. By this procedure the greens and yellows are quantitatively removed from the meal by the solvent.

The combined alcohol and acetone solution is mixed with 150 g. of coarse talc and after the lapse of an hour it is diluted with 4.5 l. of water while stirring. The chlorophyllide separates, with much admixture, in small, beautiful crystals of metallic lustre which are collected by the talc on standing for some time. Filter upon a suction filter through another thin layer of coarse talc. All the talc is washed with 0.25 l. of 55 per cent acetone and then with 0.25 l. of 55 per cent alcohol, filtered under strong suction and immediately purified further while still upon the suction filter by a very quick extraction with 1.5

l. of petroleum ether and then with 0.5 l. of ether. The two extracts, of a dirty yellowish green color, contain, besides considerable colorless substances, all the xanthophyll and carotin. After purification with methyl alcoholic potash the united extracts are employed for the fractionation of the two yellow pigments by means of methyl alcohol (see Chapter XII, section 2).

In this extraction for purifying the talc the ether finally runs off with a beautiful, light green color but it removes very little chlorophyll. The talc, which was olive brown before washing with petroleum ether and ether, has become gray and under the microscope appears full of the characteristic, small, triangular and hexagonal crystals.

The talc is finally extracted with 0.25 l. of absolute alcohol, to which 0.005 g. of oxalic acid has been added in order to prevent allomerization, and the deep green filtrate is mixed with 3–4 l. of ether. The ether is quantitatively freed from alcohol by washing 6 times with 2 l. of water each time, and is then evaporated, after drying for a short time with ignited sodium sulphate, to 1.5 l. The solution is filtered once more as quickly as possible and the ether is slowly distilled off in a water bath which is not too hot until but 20 cc. remains. Otherwise the pigment will deposit as incrustations upon the walls of the vessel. During the evaporation and on standing for a short time the ethyl chlorophyllide crystallizes beautifully. The preparation is pure, the yield amounts to 4.5 to 5 g. or about 1 g. from 1 kg. of fresh leaves; that is, more than 90 per cent of their chlorophyll content.

With laboratory equipment 2 kg. of leaf meal may be worked up in one portion and 3 charges handled in a day, not to mention the valuable yellow secondary products obtained.

Willstätter and Hug[8] obtained ethyl chlorophyllide from isolated chlorophyll by means of chlorophyllase.

A solution of 1 g. of pure chlorophyll in 200 cc. of 90 per cent alcohol was shaken for 3 days with 15 g. of enzyme material. This enzyme material had been obtained from 100 g. of dry Galeopsis leaves by exhaustively extracting the meal with alcohol and isolating the finest powder from it by sedimentation after it had stood 24 hours with a half liter of alcohol.

Under these conditions, 70 per cent of the chlorophyll was alcoholyzed as indicated by the phytol number (13.6) of the magnesium-free derivative.

[8] Ann. d. Chem. 380: 210. 1911.

The crystallized chlorophyll that was formed was isolated in a pure state; the phytol that was split off was identified analytically.

2. Methyl Chlorophyllide from Dry Leaves.[9]

In carrying out methanolysis with dried leaves unexpected obstacles had to be overcome. The enzyme is not very active in methyl alcohol that contains little water; while on the other hand chlorophyll is but poorly extracted and held in solution by methyl alcohol that contains much water. Acetone-methyl alcohol is also unsatisfactory as a solvent.

The success of the method depends upon using a mixture of acetone, water and methyl alcohol, which extracts well and in which the enzyme reaction easily runs its course. Acetone with a considerable water content extracts chlorophyll excellently; also, in the presence of water the enzyme reaction proceeds easily; the water in this mixture, however, should not be so large in comparison with the methyl alcohol that considerable hydrolysis can take place in addition to the methanolysis. Phytol was split off quantitatively in two days by the action of a mixture of 60 per cent (by volume) acetone, 10 per cent methyl alcohol and 30 per cent water upon Heracleum meal, but this was almost wholly by hydrolysis; that is, without the formation of the methyl compound.

The most suitable ratio of the solvents is a mixture of:

80% acetone (by volume),
16% methyl alcohol and
4% water.

With the usual moisture content (7 per cent) of the leaves, the liquor has the following composition:

78% acetone (by volume),
15% methyl alcohol and
7% water.

Here the ratio of the methyl alcohol to the water is 2:1, which is approximately the same as in the methanolysis of fresh leaves.

The acetone serves only as a solvent and the alcoholysis is dependent only upon the ratio of the methyl alcohol to the water.

[9] Unpublished.

Two kg. of Heracleum leaf meal are gradually stirred with a mixture of 3.2 l. of acetone and 0.8 l. of 80 per cent methyl alcohol in a powder flask, and allowed to stand, with occasional shaking, for about 40 hours, more or less, according to the enzymatic activity of the material; the end of the methanolysis is controlled by the test-tube method. For this purpose a sample is filtered off, the pigment transferred into ether and tested with 22 per cent hydrochloric acid.

When this basicity test indicates the end of the reaction, the deep green paste is filtered upon a stoneware "Nutsch" suction filter, using a filtering cloth. The meal now contains, in addition to almost all the yellow pigment, a large portion (approximately 1/3) of the crystallized chlorophyll; it is not easily extracted, particularly because much water has been absorbed. It is, therefore, extracted further upon the suction filter with 4–5 l. of acetone, alternately macerating and subjecting to the suction of a vacuum machine for about an hour. The first four liters of solvent are sufficient for the green pigments, though still more acetone is required for the complete extraction of the yellow pigments.

The total filtrate, 6–7 l., is mixed in a large vessel with 300 g. of coarsely ground talc and kept agitated by means of a broad, glass stirring rod, while 7 l. of water are slowly (in 1½ hours) allowed to flow into it from a separatory funnel. The methyl chlorophyllide crystallizes, when this is done, upon the stirrer and the walls of the vessel, as well as in the talc, in the form of lustrous, crystalline aggregates which are recognizable with the naked eye, and in single, well-formed leaflets which are mixed with the yellow and orange-red crystals of the carotinoids.

On standing two hours the liquor brightens to a pale yellow green. It is filtered through a thin layer of talc and washed with 0.5 l. of 50 per cent acetone and then with a like amount of 50 per cent alcohol. The olive brown talc is subjected to strong suction, briefly and vigorously shaken with 2 l. of petroleum ether (0.64–0.66); again brought upon the suction funnel and then washed with 1 l. of petroleum ether and 1 l. of ether. It is not feasible to undertake this important purification by simply extracting with petroleum ether upon the suction funnel, for if this is done the large amount of colorless accompanying material becomes mixed with the petroleum ether and forms a solution which when it passes through the rather thick layer of talc removes considerable chlorophyllide. On the other hand, if the colorless mate-

rial is diluted with much petroleum ether when it is shaken in the flask, only very little of the green pigment goes into solution.

The methyl chlorophyllide is extracted from the talc that has been thus purified by means of 0.8 l. of absolute alcohol which, to prevent allomerization, contains 0.02 g. of oxalic acid; it is isolated in the same manner as is ethyl chlorophyllide (see section 1).

The yields of methyl and ethyl chlorophyllide are the same; almost 5 g. from 1 kg. of dry, or 1 g. from 1 kg. of fresh leaves.

The mother-liquor from the crystallization contains, in addition to a little methyl compound, only a very little acid chlorophyllide (tested with 0.01 *N* KOH), while the isolated preparation is free from the acid chlorophyllide.

The petroleum ether-ether wash liquor has extracted from the talc all the yellow pigments that were contained in the leaves and it is the best material for their isolation.

3. Methyl Chlorophyllide from Fresh Leaves.[10]

Our method starts from the "method for working up fresh leaves" which Willstätter and Isler have described.[11]

Although it has heretofore been impossible to prevent with certainty changes in the chlorophyll during the comminution of fresh leaves, a preliminary treatment led to uniform results with different plants and greatly facilitated working up the material. It made possible the preparation of chlorophyll solutions with wholly unchanged phytochromin.

The method of Willstätter and Isler consists in using aqueous methyl alcohol of such a concentration that none of the chlorophyll is extracted. The alcohol is used in such quantity and of such strength that it is sufficiently diluted with the water content of the leaves (4/5 of their weight) so as to have little extractive action. The protoplasm is killed, the proteins are coagulated and many enzyme reactions are hindered. The leaves are thus hardened and may be ground nicely in a syenite mill without generating heat. The material is dehydrated to such an extent that in one operation it gives up its chlorophyll to the alcohol without further treatment and is sufficiently extracted.

Different plants show an interesting difference when subjected to this treatment. Some, for example, stinging nettle, plane tree and

[10] Ann. d. Chem. 387: 339. 1912.
[11] Ann. d. Chem. 380: 171. 1911.

elder, lose only slowly a small portion of their chlorophyll. The leaves become intense green in color and produce after they are centrifuged a very good yield of chlorophyll solution.

In another group of plants, typical representatives of which are Galeopsis and Heracleum, that is, those rich in chlorophyllase, the leaves are quickly bleached. An enzymatic alcoholysis results; the phytol-containing chlorophyll first goes into solution and the phytol-free chlorophyll quickly crystallizes, for the greater part in the leaves (see Chapter VII, section 2). Thus it was that when green algae (Ulva lactuca) were transported from Naples in a keg with aqueous methyl alcohol they were obtained in a bleached state, while the chlorophyll was deposited as a sediment composed of microscopical crystals of its methyl ester.

The leaves of plants plucked without their stems, that were distinguished on account of their large chlorophyllase content, were used as the initial material for methyl chlorophyllide and free chlorophyllide.

Place 20 kg. of Heracleum leaves and 32 l. of methyl alcohol in two stoneware pots with ground lids, alternately filling them with leaves and fluid. The alcohol, on account of the water content of the leaves, becomes diluted to about 66 per cent since the leaves contain only a little more than 20 per cent of dry substance. The leaves must be thoroughly wetted and must be stirred well during the treatment with methyl alcohol. The solvent is allowed to act till the leaves are wholly bleached. The time necessary for this varies with the chlorophyllase content of the crop; it usually requires 2–3 hours. The leaves are then taken out, pressed, and freed in a large centrifuge from the adhering methyl alcohol. They are then spread out in a thin layer in drying ovens and ground after they have been dried. The yield amounts to 3.5 to 3.6 kg. of light olive colored meal.

The methyl alcoholic liquors are united and stirred with about 200 g. of talc, which collects the mucilaginous, fine precipitate and makes it filter well. The filtered talc is dried in a vacuum desiccator.

Working up the Leaf Meal. It is necessary, for extraction, to work up the meal of leaves that have been treated with methyl alcohol in smaller charges (3 portions each of about 1.2 kg.) so that the chlorophyll will remain in the alcoholic solution the shortest possible time; allomerization of the chlorophyll can not be averted here by slight acidification of the alcohol, because of the amphoteric action[12] of the plant meal which absorbs acids.

[12] Ann. d. Chem. 378: 50. 1910.

The dry meal is placed upon a suction filter, tamped fast, subjected to the suction of the pump and ether (about 3 l.) is then poured on as long as it dissolves yellow pigments; at the same time this washes out many colorless substances so that a pure extract is subsequently obtained. The ether runs off at first strongly yellow in color, then yellowish green and finally pure light green; it contains almost no phytol-containing chlorophyll but a not inconsiderable quantity of methyl chlorophyllide which is removed by strong concentration and the addition of talc. It is expedient to work up this talc separately because it contains a chlorophyllide mixture which is much richer in component *b*. The meal is washed with ether, extracted with absolute alcohol and the chlorophyllide transferred into ether. The preliminary extract from 3.6 kg. of meal produced 1.25 g. of crystals.

Immediately after the preliminary extraction with ether has been made about 0.5 l. of absolute alcohol is poured upon the meal at one time. This is allowed to slowly penetrate the meal and, after a short pause, is sucked off quickly. The filtrate is then taken from the suction flask at once and is immediately transferred to ether by the addition of ether and water while, at the same time, the extraction with the pump proceeds with a further half-liter of alcohol. In all, 4 l. of alcohol are used for the extraction of 1.2 kg. of leaf powder, and the extract is transferred to 5 l. of ether. The ethereal solution was washed thoroughly six or seven times with considerable water, dried with sodium sulphate and concentrated to 2 l. The solution, in which crystallization had already begun, was filtered again and concentrated to about 100 cc. The methyl chlorophyllide separated in a fine, pure state as a loose, crystalline meal of rhombic plates. The substance contained neither yellow nor colorless admixtures and on cleavage it gave phytochlorin *e* and phytorhodin *g* exclusively, and no weaker bases. The ethereal mother-liquor is light green in color and contains a small quantity of phytol-containing chlorophyll as well as a trace of free chlorophyllide.

The yield of methyl chlorophyllide from all the meal amounted to 12.25 g. The components *a* and *b* are (as determined by means of the cleavage products, chlorin and rhodin) contained in the mixture in approximately the ratio 2 : 1. The ratio is, however, substantially altered upon fractional crystallization. The first separations are rich in *b* (for example, they contain 50 per cent of it and even more); the later ones are very rich in *a* (for example, 80 per cent).

Elaboration of the Talc Portion. The talc is given a preliminary extraction upon a small suction funnel with ether which dissolves out the yellow and other mixtures. It is then likewise extracted upon the suction funnel with absolute alcohol and the chlorophyllide is immediately transferred from the filtrate into ether. The washed and dried solution is concentrated till it crystallizes; yield 1.1 to 1.2 g.

Altogether, in this experiment 1 kg. of fresh leaves produced 0.75 g. of pure methyl chlorophyllide. The yield remained quite uniform in the elaboration of 500 kg. of Heracleum leaves.

4. Free Chlorophyllide from Fresh Leaves.[13]

Although the preparation of methyl chlorophyllide may be accomplished more quickly and economically from dry leaves, it is better to use fresh leaves for the preparation of the free chlorophyllide.

The carboxylic acid, on account of its instability, could not be isolated from the less pure, concentrated solutions such as were obtained in the enzymatic hydrolysis with leaf meal. In a concentrated solution chlorophyllide changes too easily into magnesium pheophorbide.

Even in moist, ethereal extracts of dry leaves the chlorophyll is split hydrolytically by the action of chlorophyllase and the reaction, the same as in alcoholysis, affects only a single carboxyl.

Under similar conditions hydrolysis proceeds much more quickly and smoothly with fresh leaves than does methanolysis. The leaves of plants that are rich in chlorophyllase were treated with an aqueous, indifferent solvent; namely, acetone, instead of with aqueous, methyl alcohol. The chlorophyll is hydrolyzed quantitatively and, at the same time, extracted from the leaves so that when the acetone is diluted with water the pigment precipitates in a rather pure, crystalline condition, naturally, as a mixture of the two components *a* and *b*.

24 kg. of fresh Heracleum leaves are placed with 29 l. of pure acetone in 3 stoneware jars; the water content of the acetone is brought up to about 33 per cent by the moisture content of the leaves. The leaves turn deep green immediately as a result of the passage of the chlorophyll out of the chloroplasts; even in a half hour the solvent becomes beautifully colored and the first portion of the extracted pigment already consists of free chlorophyllide. If a sample of this

[13] Ann. d. Chem. 387: 359. 1912.

extract is mixed with ether and much water an ethereal solution results which gives up all its green color to 0.01 *N* KOH. After one hour the leaves are spotted with yellow; after 3–4 hours they are light gray and completely extracted. They are softened by the acetone and not hardened as in alcohol. Chlorophyllide can not be isolated from them but is found only in the deep green, acetone solution. The solution is separated from the leaves by means of a centrifuge, mixed with much talc (500–600 g.), which is necessary for the collection of the light and fine chlorophyllide crystals, and diluted in portions with water; in all, with double the volume. During this procedure the chlorophyllide crystallizes in microscopical crystals, in regular, hexagonal plates. The liquor is decanted as much as possible from the talc, which is then filtered upon a suction funnel and dried in a vacuum desiccator. This should not take too long, else the preparation will spoil.

The dry talc is worked up in about four portions. It is placed upon the suction filter and slowly washed with much ether, which separates chiefly the yellow and colorless admixtures. When the ether starts to run off with a beautiful, light green color, it is displaced with 300 cc. of acetone, which dissolves the chlorophyllide with extreme ease. The substance is transferred from the acetone solution into ether by mixing it with water (distilled water should always be used with the free chlorophyllides as otherwise green floccules of calcium salts would precipitate) and ether (2 l. for each portion). The ether is wholly freed from acetone by washing 8–10 times and is nearly dried with sodium sulphate. The solution must be kept dilute so that during the time required for the necessary procedures the spontaneous decomposition of the chlorophyllide, which has been mentioned before, does not occur. The complex compound is more stable in weaker concentrations although a somewhat brownish precipitate is usually deposited. The ethereal, chlorophyllide solution is very quickly and very strongly concentrated, the process of concentration being interrupted by repeated filterings till the paper is no longer colored brown. Finally, on evaporating almost to dryness, the chlorophyllide crystallizes in leaflets which are always hexagonal, shows a splendid luster and appears blue black by reflected light and green by transmitted light. Crystallization takes place more easily in an ethereal solution that contains a little water than in one that has been thoroughly dried.

The yield amounted to 12 g., that is, 0.5 g. for each kg. of fresh leaves. The preparation gives a pure green, ethereal solution, which

is totally decolorized by 0.01 *N* KOH. It is free from yellow substances as well as from esters. In the cleavage test, phytochlorin *e* and phytorhodin *g* are obtained in the ratio 3 to 1 with none of the weak bases.

The chlorophyllide is more easily precipitated from an ethereal solution that is moderately concentrated (200 cc. for each portion) by the slow addition of low boiling petroleum ether (300 cc.); it quickly separates as a very fine, crystalline powder of bluish to greenish black color, which under the microscope shows only the form of spherical aggregates. This form of the preparation is, however, less stable than the crystalline; in four months one-third of it had changed into magnesium pheophorbide.

Hydrolysis of methyl chlorophyllide. Even pure methyl ester may be hydrolyzed with the aid of chlorophyllase though with much more difficulty than crude chlorophyll. For this experiment, the enzyme was prepared from 50 g. of the meal of Heracleum leaves by repeated thorough extraction with 80 per cent acetone. The decolorized meal was agitated with 50 cc. of water and transferred to a solution of 0.5 g. of methyl chlorophyllide *b* in 200 cc. of pure acetone. After the addition of a small quantity (0.02 g.) of oxalic acid the solution was allowed to stand for four days with frequent shaking. After two days, however, the reaction did not proceed any further. The free chlorophyllide was quantitatively isolated from the product of the reaction by the use of ammonia after transferring to ether and removing the acetone by washing. The ethereal solution was mixed with a third of its volume of petroleum ether and the salt was precipitated upon talc by the introduction of gaseous ammonia. By agitating with ether and water and the addition of a few drops of mono-sodium phosphate solution, free chlorophyllide *b* was isolated with a yield of only 0.05 g. It showed the red phase and gave pure phytorhodin *g*.

X. ISOLATION OF THE CHLOROPHYLLIDE COMPONENTS A AND B.

The various preparations of crystallized chlorophyllides are often distinguished by the ratio of chlorin to rhodin obtained in the cleavage test. In the preparation of methyl chlorophyllide the talc portion contained a preponderance of the *a* component. In working up certain plants; for example, Ulva lactuca and Aesculus hippocastanum, a preponderance of *b* methyl chlorophyllide was frequently isolated, but a single component never appeared alone.

This rather fortuitous fractionation by crystallization is therefore not relied upon as a means for the separation of the components. The pure compounds *a* and *b* are isolated from the chlorophyllide mixtures according to the Stokes and Kraus principle of fractionation but, on account of the specific solubility ratios of the chlorophyllides, use is made of a new system of solvents. Since the compounds are insoluble in petroleum ether and carbon disulphide, a difference in their solubilities in ether is made use of in the separation; *b* is more difficultly soluble in it than *a*. The substances are distributed between ether and aqueous methyl alcohol but, instead of ether which is still miscible with strongly aqueous methyl alcohol, a mixture of equal volumes of ether and petroleum ether is used. The component *b* is more easily extracted from this by aqueous methyl alcohol. Methyl chlorophyllide *b* is extracted, therefore (from the ether-petroleum ether solution of the component mixture), by means of 50–60 per cent and free chlorophyllide *b* by means of 40–50 per cent methyl alcohol.

1. Separation of the Methyl Chlorophyllides.[1]

Sixty per cent methyl alcohol is most suitable for the fractionation; with its use almost pure methyl chlorophyllide *b* is removed from the ether-petroleum ether solution, especially in the first extractions. If the methyl alcohol were more dilute it would extract too little material from the ether. More concentrated methyl alcohol does not differen-

[1] Ann. d. Chem. 387: 345. 1912.

tiate so well since it removes too much of the *a* component along with the *b*.

The purity of methyl chlorophyllide preparations is endangered by the fact that chlorophyll is easily allomerized through the action of alcohols. In order to prevent this, all methyl and ethyl alcohols or aqueous alcoholic solutions used by us have a small amount of oxalic acid added, namely, 0.001 per cent. Decomposition of the magnesium compounds is not observed with this dilution of the acid.

The manner of fractionation varies somewhat, depending upon whether the mixture is poorer or richer in *b*; hence two examples follow.

A. Separation of a mixture of about 3/4 *a* and 1/4 *b* component. With a 7 l. separatory funnel 2 g. of the initial material may be worked up at a time and, at most, two such charges can be run in a day. The solvents employed must be pure; in order to free the Kahlbaum ether from fat it was redistilled. In spite of the greater cost it is advantageous to employ a petroleum ether fraction which boils at 30–50°.

Two g. of methyl chlorophyllide were dissolved in 0.3 l. of absolute ethyl alcohol, since solution in ether or methyl alcohol would be too tedious, and the solution is immediately mixed in a large separatory funnel with 2.8 l. of ether. The ethyl alcohol is then washed out by shaking three times, with a liter of water each time, whereupon the volume of the ethereal layer diminishes to 2.5 l. In order to avoid the precipitation of the chlorophyllide by petroleum ether 0.3 l. of methyl alcohol are now added and then, while shaking, 2.5 l. of low boiling petroleum ether. For the first extraction 1 l. of 60 per cent methyl alcohol is used and, since the solution already contains 300 cc. of methyl alcohol, 200 cc. more of water are used. The methyl alcoholic layer takes up fully 5 per cent of the pigment, which consists of about 70 per cent *b* and 30 per cent *a*. It is colored a yellowish green; a test sample, on transferring to ether and saponification with methyl alcoholic potash, gives the brown phase with a strong red tint. After this first fractionation, the volume of the ether-petroleum ether is marked and kept constant in the following eleven extractions by the addition of ether alone. The second to the twelfth fractionations are each carried out with 1 l. of 60 per cent methyl alcohol. Then, without replenishing the ether, further extraction is carried out with 60 per cent methyl alcohol, usually about 5 times. From about the fifteenth fractionation on, tests (phase test) are again made to ascertain whether

b has been completely removed; that is, whether, finally, methyl chlorophyllide *a* alone is passing into the methyl alcoholic layer. These are made by transferring samples from the alcoholic solution (no longer yellowish tinted but pure green) into ether and mixing with methyl alcoholic potash. When the green color no longer changes to brown but to a pure yellow, *a* alone is present. Then, by frequent shaking with considerable water (or by allowing a stream of tap water to flow through the separatory funnel) the methyl alcohol is completely removed, and also the ether to a great extent. As a consequence of its insolubility in petroleum ether the methyl chlorophyllide precipitates as steel blue, crystalline floccules. The aqueous layer is run off, and the liquor is then mixed with some ignited sodium sulphate and a little talc (about 50 g.) which wholly decolorizes it, after which it is filtered. It is now washed with ether and extracted immediately. The preparation would suffer decomposition if allowed to stand in this condition upon the "Nutsch." The methyl chlorophyllide is extracted with absolute alcohol, from which the chlorophyllide is transferred into ether (about 1.5 l.). The ethereal solution, freed from alcohol by thorough washing, is, after it has been dried, concentrated to about 80 cc. During the evaporation the methyl chlorophyllide *a* crystallizes in beautiful aggregates of rhombic plates of blue luster.

The yield of *a* amounts to 0.6 to 0.7 g.; the material requires no further purification.

The methyl alcoholic solutions should be worked up at the same time for the *b* component, using the first eight extractions. They are combined in twos. The combined first and second extracts are mixed with 0.5 l. of methyl alcohol so that the solution consists of about 65 per cent methyl alcohol; the same procedure is carried out with the third and fourth extracts, while the fifth and sixth, and the seventh and eighth should be used in their original concentrations. The four pairs of extracts are extracted, first, each with one liter, and then, each with 0.5 l. of an ether-petroleum ether mixture (3 volumes of petroleum ether and 1 volume of ether) in order to separate the *a* component as much as possible. The upper layer gives with the "phase test" a pronounced yellow, while the lower (after transferring the substance from 5 cc. of the methyl alcoholic solution into ether) gives a reddish brown phase with methyl alcoholic potash. The complete separation of *a* in a single procedure did not occur with initial material that was poor in *b*; such material gave a better yield of *b* if it was first worked

up to a preparation containing 90 per cent of *b*, which was then purified by a process of fractional precipitation.

For the isolation of crude methyl chlorophyllide *b* the methyl alcoholic extracts that have been thus washed are united and extracted with ether; the ethereal solution is wholly freed from methyl alcohol by thorough washing and is then concentrated to 0.5 l. The material is not permitted to crystallize, however, since it is advantageous to separate still more of the *a* component by precipitation. For this purpose the still warm, ethereal solution is mixed with a little talc (about 30 g.) and 300 cc. of petroleum ether. Practically nothing but a little of the *a* component remains in the mother-liquor. The talc is filtered upon the "Nutsch" and washed with ether until this, as it runs off, has only a pale yellowish green color and shows, on mixing with methyl alcoholic potash, a beautiful color change to brownish red. The ether, at first, removed some of the methyl chlorophyllide *a*. The methyl chlorophyllide is removed from the talc at once by extraction with absolute alcohol, transferred to ether, washed and concentrated to 50 cc.; a yield of 0.25 to 0.3 g. is obtained. It forms beautiful, greenish black crystals with a metallic luster, and consists of about 90 per cent of component *b* and 10 per cent of *a*.

Purification of Methyl Chlorophyllide b. The fractional precipitation which has already been employed once for purposes of isolation is repeated three times as a means of purification.

One g. of the 90 per cent methyl chlorophyllide is dissolved in 600 cc. of absolute alcohol and an ethereal solution is made from it by mixing it with 4 l. of ether and washing out the alcohol; to do this it is necessary to shake 6 times, with 1.5 l. of water each time. The ethereal solution is concentrated to 600 cc., treated while still warm and before crystallization begins, with about 50 g. of talc and precipitated with 400 cc. of petroleum ether. The talc is filtered upon a "Nutsch" and washed thoroughly with ether; in this procedure some of the *b* is lost but *a*, particularly, is removed. The talc upon the "Nutsch" is then immediately extracted with absolute alcohol (about 0.5 l.) in order that the pigment may be transferred to ether again and again separated, after the addition of talc, by means of petroleum ether. The same fractional precipitation is carried out a third time. The first two ether-petroleum ether mother liquors, after they had been filtered from the talc, were blue green and contained the *a* component; the third mother liquor, on the contrary, was colored a pale

yellow green. Perfectly pure methyl chlorophyllide *b* is obtained a a very beautiful, dark green crystallization by concentrating th ethereal solution that has been prepared for the fourth time. It i homogeneous and consists of rhombic plates which have a metalli luster; the mother liquor is very light in color. The yield amounte to 0.5 to 0.6 g., or a little more than half of the 90 per cent prepara tion used. With crude products that contained smaller percentage of methyl chlorophyllide *b* (70–80 per cent), repeated (in this case five times) fractional precipitation gave just as good results.

B. Separation of a mixture of about 60 per cent methyl chloro phyllide *a* and 40 per cent *b*. If the initial material is richer in *b*, i is easily possible to get a nearly pure preparation of *b* (95–98 pe cent) in one procedure. A greater number of methyl alcoholic extract may then be used and they become so much richer in *b* that they ma be sufficiently freed from *a* by shaking with ether-petroleum ether an a single precipitation.

Two g. of methyl chlorophyllide are brought into ether after inter mediate solution in alcohol. The first resolution into component part is effected with 0.6 l. of methyl alcohol, 2.5 l. of petroleum ether an 0.4 l. of water; each of the following extractions, with 1 l. of 60 pe cent methyl alcohol. The volume of the ether-petroleum ether laye is kept constant until toward the end of the fractionation. The firs 12 extracts are worked up in pairs. The first two pairs are brough back to a higher concentration (65 per cent) by the addition of 0.5 l of methyl alcohol, the 5th and 6th extracts are mixed with only 0.3 l the 7th and 8th with 100 cc. of methyl alcohol while those that follo are used in their original concentrations. The extracts are the washed but once with 1 l. of petroleum ether-ether (3 : 1); the *a* com ponent that accompanies the methyl alcohol is very thoroughly ex tracted from it again as may be seen by the color of the solution an the color change with methyl alcoholic potash. Only the first 1 methyl alcoholic extracts are worked up for *b* as the others contai too little pigment. The methyl chlorophyllide *b* is transferred t ether from the aqueous methyl alcohol and, as described in A, frac tionally precipitated once from a concentrated ethereal solution wit petroleum ether; it is dissolved again and brought to crystallization.

For the purification of the *a* component it is necessary to continu the washing with 60 per cent methyl alcohol 6–10 more times. Finall no more *b* can be detected in the extracts. The methyl chlorophyllid

a is, as detailed above, isolated by the aid of talc and then obtained in crystalline form from its ethereal solution.

The yield and the purity of *a* depend upon the number of extractions. In working up 100 g. of methyl chlorophyllide mixture there was obtained from each 2 g., generally, 0.65 g. of 95 per cent or 0.5 g. of pure methyl chlorophyllide *a* and, in addition, 0.45 to 0.55 g. of methyl chlorophyllide *b*.

2. Separation of the Free Chlorophyllides.[2]

The free carboxylic acids are separated by means of more dilute methyl alcohol than are the methyl compounds.

Since they allomerize even more easily than do their methyl and ethyl esters—simple solution in alcohol suffices to bring about this transformation—the addition of 0.001 per cent of oxalic acid is especially important in their isolation. Besides, spontaneous decomposition involving the splitting off of magnesium occurs very easily in the case of the acid chlorophyllides; all operations should, therefore, be carried out very quickly.

Two g. of a crystalline chlorophyllide mixture are dissolved in acetone and poured into 3 l. of ether; the acetone is mostly removed by shaking with water three times. The ethereal solution is then mixed with 0.5 l. of methyl alcohol and 2 l. of petroleum ether (B. P. 30–50°) and separation is effected by the addition of a half liter of water. After vigorous shaking, the yellow-green, dilute methyl alcoholic layer, which contains a preponderance of component *b*, is allowed to flow from the separatory funnel into a second funnel containing ether. By dilution with water an ethereal solution of crude chlorophyllide *b* is obtained.

The volume of the ether-petroleum ether layer that remains is now marked and kept constant from now on by the addition of ether after each fractional separation. Two more extracts, each with 1 l. of 50 per cent methyl alcohol, contain enough of the *b* component to be transferred to ether in the same manner and worked up together with the first extract. The washed, ethereal solution is concentrated to 300 cc. and precipitated with 600 cc. of petroleum ether upon talc. After washing with petroleum ether and a little ether an exhaustive extraction with ether is made upon a "Nutsch" and 0.25–0.30 g. of chloro-

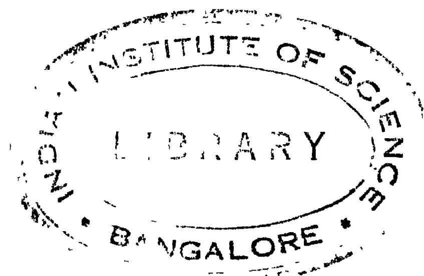
[2] Ann. d. Chem. 387: 362. 1912.

phyllide, with a content of 2/3–3/4 of *b*, are precipitated from the concentrated solution by means of much petroleum ether.

The purification of component *a*, which has accumulated in the petroleum ether-ether, should be carried out at the same time as the working up of the first 3 methyl alcoholic extracts for *b*. We shake five more times, with 1 l. of 50 per cent methyl alcohol, each time replenishing the upper layer with ether, and, finally, four times without the addition of ether. The ether-petroleum ether solution, which at first was pure green, now has a blue green color, which is, in fact, practically blue, when the alcohol is washed from a test sample. The end of the fractionation can not be easily recognized by means of the phase test in the case of the free chlorophyllides. Finally, by washing with distilled water, the methyl alcohol and so much ether are washed from the petroleum ether layer that the chlorophyllide begins to crystallize on the walls of the separatory funnel. The separation is completed in the same vessel by the addition of another liter of petroleum ether as well as some sodium sulphate and about 100 g. of talc. The talc is first washed with petroleum ether upon the "Nutsch," then with a little ether in order to displace the petroleum ether, and immediately extracted with more ether. The solution that is obtained is concentrated to a few hundred cubic centimeters and filtered, then evaporated to 100 cc., and precipitated by the slow introduction of 400 cc. of petroleum ether. The deposit (0.55–0.60 g.) formed a dull, bluish black powder which showed a crystalline structure under the microscope and frequently consisted of rounded plates. In five experiments it contained the pure component *a* without any admixture of *b*; in one experiment, in which the number of extractions with methyl alcohol had been decreased, it contained a few per cent of *b*.

The purification of component *b* consists of a repetition of the fractionation method. 0.45 g. of a preparation that is rich in *b* (75 per cent) is dissolved with 100 cc. of methyl alcohol in 1 l. of ether and mixed with 600 cc. of petroleum ether. For the fractional separation this was shaken with 100 cc. of water and an additional 500 cc. of 40 per cent methyl alcohol. The dilute alcoholic layer was decidedly yellow green in color. It was run off directly into ether, to which the material was completely transferred by the addition of water. The ether-petroleum ether was then shaken three more times with 0.5 l. of 40 per cent methyl alcohol, each time replenishing the volume of the upper layer with ether. These three methyl alcoholic extracts

were likewise extracted with ether and all the ethereal solutions of chlorophyll *b* combined. After the methyl alcohol was removed as quickly as possible by washing, the united ethereal solutions were evaporated to 300 cc. and the substance again precipitated upon talc by the addition of 600 cc. of petroleum ether. It may be extracted from the talc upon a "Nutsch" by 1 l. of ether. By concentration to 50 cc. and slow mixing with petroleum ether, the pure carboxylic acid was finally separated as a dark green, micro-crystalline precipitate in a yield of 0.15 g. No phytochlorin *e* could be detected in this preparation by means of the cleavage test.



XI. DESCRIPTION OF THE CHLOROPHYLLIDES.

1. Crystallized Chlorophyll.[1]

Crystallized chlorophyll is a mixture of the ethyl chlorophyllides *a* and *b*. They form, in varying proportions, isomorphous crystals but are generally obtained in the usual component ratio $a : b = 2.5 : 1$. With variations of the content of the two components, certain properties, especially the optical properties, also vary.

Crystallized chlorophyll shows all the characteristics and reactions of the natural pigment if we disregard the peculiarities that depend upon its phytol content. Substitution of the ethyl radical for the phytol group causes the pronounced ability to crystallize and the desirable decrease of solubility in various solvents.

The molecule decreases about 28 per cent in weight as a result of the change in its ester group, namely, from 906.6, the probable average for the normal mixture, to 654.6; for the investigation of phytochromin, ethyl chlorophyllide is to be regarded, therefore, as unaltered 139 per cent chlorophyll, so to speak. One g. of chlorophyll is equivalent in color to 0.72 g. of crystallized chlorophyll. Crystallized chlorophyll forms, for the most part, sharply defined, equilateral, three- and six-cornered plates (Plate II, Fig. 1 and 2).

Occasionally, by a truncation of the corners of the three-cornered crystals, hexagons, having sides of unequal length, occur. Besides these typical forms, wedge-shaped and blunt lancet-shaped prisms also occur. Very blunt surfaces of a vicinal rhombohedron, whose edges are perpendicular to three lateral edges of the hexagon, are often observed. The hexagonal system probably appears here in a trigonal hemihedrism. The diameter of the crystals is usually 0.1–0.2 mm.

The crystalline powder is blue black and is distinguished by a bright, metallic luster. In the sunlight, especially, it shows wonderful reflections. The powder is dark green in color. In transmitted light the plates appear now more blue green, then yellow green.

On heating, the material decomposes with intumescence and the development of vapors, without exhibiting a melting point. The vapors

[1] Ann. d. Chem. 358: 277. 1907, and Ann. d. Chem. 382: 151. 1911.

slightly redden a pine splint that has been soaked in hydrochloric acid. A carbon that is difficult to burn is formed and, finally, pure white magnesia remains.

The usual ethyl chlorophyllide mixture is easily soluble in absolute alcohol, wood alcohol and acetone, insoluble in petroleum ether, rather difficultly soluble in ether and a mixture that is rich in *b* is even very difficultly soluble in ether (1 g. in 2.5 l.). The ethereal solution is blue green while the alcoholic solution is much more yellowish green. Chloroform dissolves it easily, especially when hot; boiling benzol, rather easily, but when cold with difficulty; methylal, easily on boiling and only with slightly more difficulty when cold. Veratrol was a suitable solvent for the determination of its molecular weight[2] by the cryoscopic method. The chlorophyllide may be beautifully recrystallized from methylal, as well as from ether, especially by transferring it to the latter with some alcohol that contains a little oxalic acid; especially beautiful crystals may be obtained from 90 per cent alcohol in which a trace of oxalic acid has been dissolved.

Without this addition of oxalic acid, ethyl chlorophyllide undergoes complete allomerization in ethyl or in methyl alcohol, at most in several hours. It consequently becomes uncrystallizable and is easily soluble in ether; it then no longer possesses the brown phase.

On heating the chlorophyllide with concentrated nitric acid a clear yellow solution is produced. It is distinguished from the phytyl compound in that no oil separates here.

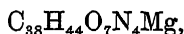
Ethyl chlorophyllide is indifferent, having neither acid nor basic properties. Twenty per cent hydrochloric acid does not dissolve the solid substance, but the greater part of the pigment is extracted from its ethereal solution, with the loss of magnesium, by 20 per cent hydrochloric acid and it is quantitatively extracted by 22 per cent HCl. The simple chlorophyllide is rather sharply distinguished from chlorophyll by this basicity test.

By means of cleavage with acid a mixture of *a* and *b* ethyl pheophorbides arises, which may be separated from ether into two decidedly different fractions, which differ in solubility, color and crystal form. The first crystals that separate consist of black, shiny, rhombic plates, rich in component *b*; then, long, reddish violet-gray needles, consisting predominantly of component *a*, separate.

[2] Ann. d. Chem. 382: 155. 1911.

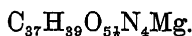
2. Method of Analysis of Compounds with a Large Molecular Weight.

Ethyl chlorophyllide, dried (1.25 per cent loss in weight) over phosphorus pentoxide in a vacuum desiccator, which had been evacuated with a water jet vacuum pump, corresponded to the formula:



$$C_{38}H_{44}O_7N_4Mg,$$

but the analytical result was misleading. At 105° C. and 0.001–0.01 mm. pressure the substance suffered a further loss of 5 per cent in weight and then agreed with the formula:



$$C_{37}H_{39}O_{5\frac{1}{2}}N_4Mg.$$

Such a difference of an atom of carbon is of great significance in clearing up the relations that exist between the members of so complicated a group. The second formula would indicate that the chlorophyllide consists of:

1. Chlorophyllide *a*, $C_{37}H_{39}O_{5\frac{1}{2}}N_4Mg$,
2. Chlorophyllide *b*, $C_{37}H_{37}O_{6\frac{1}{2}}N_4Mg$.

Since the amounts of *a* and *b* are approximately in the proportion of 2.5 to 1.0 the admixture of *b* has very little influence on the composition of the preparation. Each of the two components is regarded as consisting of approximately equal parts of lactam and lactam hydrate, probably united as half-hydrates.

In spite of the size of the molecule, it may be concluded as a consequence of elementary analysis that there is a probable difference in the carbon content in the two dry conditions. Also the carbon of the mate-

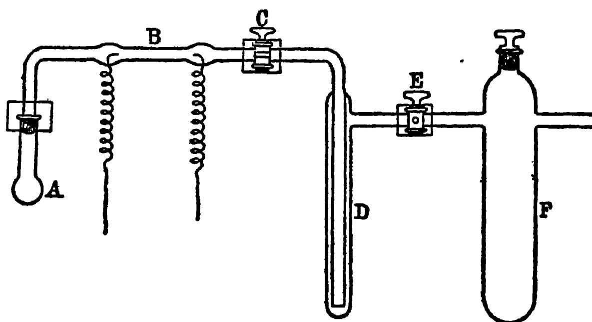


Fig. 9.

rial, when dried at 105°, was not increased to the extent it would have been had water been given off. Not only water, but also a carbon compound, was therefore lost in drying. Examination showed that it was not a question of formaldehyde, which could have been thought of, nor of carbonic acid or methyl or ethyl alcohol, but that ether escaped along with the water (with only a trace of alcohol).

The small drying flask A (Fig. 9) is connected through the discharge tube B with a condensation receiver D, a vessel F which contains animal charcoal, and a high vacuum pump. The connections are short and large; the ground joint of the small drying flask and the stopcocks, which have 5 mm. holes, are made tight by imbedding them in paraffin. When the vacuum has been obtained, the connection to the pump is shut off and the vacuum is increased by cooling the animal charcoal with liquid air until the electric discharges disappear. The stopcock E is then closed and the condensation receiver is placed in liquid air. The small flask is placed, for the drying, in the atmosphere of a V. Meyer toluol bath which has been filled with iron filings; drying requires 6–8 hours. The stopcock C is then closed, the connections between A and C and between E and F are cut and the condensate receiver, for example, is inserted in a combustion tube.

The condensates showed the atomic relation:

1. 1 C : 7.45 H : 2.65 O.
2. 1 C : 6.51 H : 2.37 O.

In this way we found a little more water and somewhat less ether (2 per cent of the weight of chlorophyllide as ethyl ether) than corresponds to the elimination of 1.25 moles of H_2O and 0.25 mole of ether.

The condensate is not alcohol. It does not form esters with benzoyl chlorides, but on heating with hydriodic acid it forms ethyl iodide.

Vacuum drying is very important, also, for the analysis of many other chlorophyll derivatives, especially on account of their strong union with ether. The etherates, especially of the magnesium compounds, the phyllins, behave on drying similarly to the Grignard magnesium compounds;[3] the crude products not so much so as preparations that have been recrystallized from ether. Glaucophyllin and rhodophyllin crystallize with an ether content of 14 per cent and pyrrophyllin with 11 per cent. The ether is combined so strongly that a water jet pump vacuum and a temperature of 140–150° must be employed for

[3] See B. E. E. Blaise, Compt. rend. 132: 839. 1901.

3–5 months in order to remove it; the sensitive substances may be decomposed by such a procedure as this.

In most cases, drying or the removal of the ether is facilitated by the use of reduced pressures; in such cases heating on a toluol bath is sufficient. Some of the phyllins mentioned are exceptions, for with them even a high vacuum did not particularly accelerate the drying.

We used for many years a Rheden mercury pump, capable of producing a vacuum of 0.001–0.03 mm. in the preliminary preparation of the material for analysis. Instead of this we now use a pump aggregate made by "Leybold Nachfolger." This consists of a Gaede mercury pump and a rotary pump and is superior to the former pump with respect to suction effect and the vacuum that may be attained.

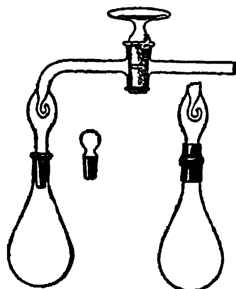


Fig. 10.
Drying bulbs.

The insertion between the substance and the mercury pump of a tube that contains gold leaf is recommended, especially when volatile constituents are to be condensed. Most compounds that contain water and ether are dried to constant weight in a few hours or at least in a few days when heated and subjected to the action of this pump.

After drying in this way the substances are often very hygroscopic. For elementary analysis, therefore, they are never weighed in the open but the quantities taken are determined by the differences in the weights of the drying bulbs before and after they are poured out. The pyriform shape (Fig. 10) of the drying bulb is important in order that the substance may be shaken into the boat without any loss. The unavoidable absorption of moisture is still to be considered. During the interval required till the boat is introduced into the combustion tube 2–3 mg. of water are usually absorbed by the substance; this is

determined and subtracted from the water that is found upon combustion.

These practices are to be observed in the analysis of other high molecular weight compounds also, and we recommend that the composition of the constituents that escape on drying be investigated by means of their condensation in the same manner as has been done in the case of crystallized chlorophyll and the phyllins. Because of the general significance of the method, still another example is quoted; the analysis of a metal-free compound of the hemin group.

Willstätter and M. Fischer removed the iron from hemin by the action of liquid hydrochloric acid (see Chapter XXIV, section 2); the chloride that was formed produced in hot glacial acetic acid solution, on treatment with sodium acetate and precipitation with water, a new compound of the hematoporphyrin group.[4] The composition of the desiccator-dried substance was identical with that of hematoporphyrin as determined by the analyses of Nencki and Sieber[5] and according to our own determinations. A formula of 6 carbon atoms, therefore, appeared appropriate for it, probably $C_{33}H_{38}O_6N_4$

Found	Calculated for $C_{33}H_{38}O_6N_4$[a]
C 66.93	67.54
H 6.47	6.53

Hematoporphyrin, according to Nencki and Sieber (averaged)
C 66.89
H 6.35

After drying at 105° in the vacuum produced by the Gaede pump the preparation contained only 4 atoms of oxygen.

Found	Calculated for $C_{33}H_{34}O_4N_4$
C 71.70	71.96
H 6.48	6.23

The result of the analysis indicates that 2 molecules of water have been split off. The loss on drying did not amount to 6.1 per cent as calculated for it but was double that. Hence, a carbon compound was split off.

2.4 g. lost 0.26 g. The vapors were condensed in a receiver (a test tube with a lateral addition and a lead-in tube) that was cooled with liquid air. The condensate contained 0.21 g. of acetic acid.

[4] Paper XXIII.

[5] Nencki. Opera II, 79.

[a] Trans. Calculation shows that this should be 38 not 28.

The preparation gave up 1 mole of acetic acid and 1 mole of water when it was dried. The composition of the desiccator-dried substance is, therefore,

$C_{35}H_{40}O_7N_4$, which, by calculation, contains C 66.84, H 6.42.

3. Determination of the Methyl and Ethyl Groups when Present Together.

The discovery of the methyl and ethyl groups that are present in crystallized chlorophyll was fundamental to our knowledge regarding chlorophyllase and crystallized chlorophyll. The investigation was, at first, led astray because of an error in the method of procedure. According to the methoxyl determination by Zeisel's method crystallized chlorophyll gave values that corresponded to two methyls; besides, by following the instructions of F. Feist[6] methyl iodide was identified in the form of trimethylphenylammonium iodide with the help of an alcoholic solution of dimethylaniline But in order to determine the alphyl iodide by means of an aromatic base, an alcoholic solution of the latter must not be employed, since ethyl iodide has scarcely any action upon it. The method was not tested for the detection of ethyl. Methyl iodide is found by means of it while ethyl iodide is overlooked. Undiluted dimethylaniline, on the contrary, is suitable for the detection of both and a 10 per cent alcoholic solution of trimethylamine is still better.

By means of this reagent it was learned that ethyl chlorophyllide that had been dried in a high vacuum at an elevated temperature splits off methyl and ethyl iodides in equimolecular proportions when it is heated with hydriodic acid.

The determination of a mixture of both alphyl iodides[7] depends upon the following observations on the yields of quaternary ammonium salts that are obtained from them upon the addition of dimethylaniline and trimethylamine; 0.5 g. of alkyl iodide was mixed each time with 10 cc. of dimethylaniline or with 10 cc. of a 10 per cent solution of dimethylaniline or trimethylamine at 20°.

1. When dimethylaniline is used, the separation of the iodides may be quite satisfactorily based upon the different speeds of reaction of the alphyl iodides or upon the different solubilities of the quaternary iodides, especially in chloroform.

[6] Ber. d. d. chem. Ges. 33: 2094. 1900.

[7] Ann. d. Chem. 382: 148. 1911.

Hours	With methyl iodide			With ethyl iodide		
	1	6	24	1	6	24
Dimethylaniline, in alcohol	4	26	64	0	0	4
Dimethylaniline, undiluted	46	95	100	1	8	31
Trimethylaniline, in alcohol	98	99	99	9	77	90

Trimethylphenylammonium iodide (M. P. 212°) is very difficultly soluble in chloroform at room temperature and difficultly soluble in this solvent when boiled. It is rather difficultly soluble in warm acetone and in cold alcohol.

Dimethylethylphenylammonium iodide (M. P. 136°) is easily soluble in chloroform, quite easily so in warm acetone and easily so in cold alcohol.

2. On the basis of the following solubility determinations the separation is more sharply and easily performed by means of trimethylamine.

	Tetramethyl-ammoniumiodide	Trimethylethyl-ammoniumiodide
Water	difficultly soluble	extremely easily soluble
Acetone, chloroform	soluble in traces	appreciably soluble
Absolute alcohol	very difficultly soluble; when hot, 1 g. in 1060 g.	cold, easily soluble; hot, 1 g. in 1.23 g.

Absolute alcohol is especially suitable for a quantitative separation.

Example: 1.18 g. of crystallized chlorophyll furnished 0.60 g. of iodide mixture. The separation gave: 1. 0.29 g. of pure tetramethylammonium iodide; 2. 0.02 g. of a mixture; 3. 0.23 g. of trimethylethylammonium iodide.

The method fills a great need; in general, it is suitable for the determination of methyl and ethyl alcohol in the presence of each other.

4. Methyl Chlorophyllides a and b.[8]

Methyl chlorophyllide a,



$$(C_{32}H_{30}ON_4Mg)\ (COOCH_3)_2 + \tfrac{1}{2}\ H_2O,$$

crystallizes in sharply defined, rhombic-shaped leaflets (Plate II, Fig. 3) which are mostly united in aggregates; also frequently in predomi-

[8] Ann. d. Chem. 387: 351–355. 1912.

nantly prismatic shaped crystals. By transmitted light under the microscope the thin crystals appear green, the thicker ones blue green; the powder is bluish black. The preparation is stable in the air and may be preserved as long as it is desired in the crystalline condition.

The *a* component dissolves in alcohol very easily with a green color and blood red fluorescence; in dry ether with difficulty; namely, 1 g. of desiccator dried material in 760 cc. at 19°; in moist ether more slowly than in anhydrous ether. The color is blue green in ether; much more blue than in the alcohols. Methyl chlorophyllide *a* is easily soluble in acetone, very slightly soluble in benzol and carbon disulphide, insoluble in petroleum ether and easily soluble in pyridine.

For recrystallization, powdered methyl chlorophyllide is dissolved in absolute alcohol and transferred to ether. If the ether which contains alcohol is poured in a layer over water, a portion of the substance gradually crystallizes. With larger quantities, the ethereal solution is concentrated after the alcohol has been thoroughly washed out; if any allomerized product is formed in this procedure it will all remain in the ethereal mother liquor.

Methyl chlorophyllide *a* is chemically indifferent; it absorbs no ammonia gas. It is sensitive toward acids; a dilute ethereal solution changes slowly to brown when it is shaken with 5 per cent hydrochloric acid; with 10 per cent hydrochloric acid it changes quickly.

Methyl chlorophyllide b,



$$(C_{32}H_{28}O_2N_4Mg)(COOCH_3)_2 + \frac{1}{2}H_2O,$$

crystallizes in sharply defined, rhombic shaped plates (Table II, Fig. 4) which are similar in form to crystals of *a* but have another color. The crystals are greenish black in reflected light, and in transmitted light under the microscope they are yellow, olive green and olive brown, according to the thickness of the crystal. The powder is greenish black. Even with mixtures of the two chlorophyllides their content of the components may be estimated; the more *a* they contain, the more the blue color appears; with an excess of *b* the dark green color shows more.

The solubility of methyl chlorophyllide *b* is less than that of *a*, especially in ether. It is very difficultly soluble in this, only 1 g. in 2.8 l. at 19°. It is also difficultly soluble in benzol. In absolute alcohol it dissolves very easily with a yellowish green color and a brownish red fluorescence, while the ethereal solution is pure green.

In the phase test with the ethereal solution there occurs immediately a change to a beautiful red; it is brownish red only if *a* is admixed, and a turbid or reddish brown if the substance has deteriorated as a result of the action of alcohol. The red phase soon changes to a brownish red and then, much more slowly than with *a*, over to yellowish green. Methyl chlorophyllide *a*, on the other hand, gives in the test a color change to pure yellow. In an alcoholic solution, especially in one containing water, the phase passes away much more quickly.

The *b* chlorophyllide is somewhat more stable than the *a* toward acids; the ethereal solution retains its color for some time on shaking with 10 per cent hydrochloric acid. The magnesium is not split off quickly unless a concentration of 15 per cent acid is reached.

5. The Two Free Chlorophyllides.[9]

Chlorophyllide a, $(C_{32}H_{30}ON_4Mg)$ (COOH) $(COOCH_3) + \frac{1}{2} H_2O$, may be recrystallized from aqueous ether or it may be precipitated in a crystalline form from acetone by the addition of water. Only one crystalline form was observed, hexagonal plates, which are bluish black with a metallic luster in reflected light; they are green to bluish green in transmitted light. In ethereal solution, the color of free chlorophyllide *a* is bluer than that of the methyl compound; its alcoholic solution on the other hand is pure green; the fluorescence is dark red.

The acid dissolves very easily in absolute alcohol and acetone; slowly, but still very easily, in 96 per cent alcohol; rather difficultly in ether (the crystallized material more easily than the precipitated); it is insoluble, on the contrary, in cold benzol and petroleum ether.

One free carboxyl suffices to make the chlorophyllide sufficiently acid to be abundantly extracted from an ethereal solution (0.02 g. in 100 cc) by 0.001 *N* KOH; 0.0005 *N* KOH extracts only a little of the acid from the ether; 0.00025 *N* extracts none. The substance distributes itself between 0.01 *N* Na_2CO_3 and ether; 0.001 *N* soda dissolves only a very little. Sodium bicarbonate (5 per cent) does not extract the carboxylic acid from ether. Ammonia (0.5 *N*) completely extracts the chlorophyllide from ether, 0.05 *N* abundantly, 0.005 *N* still noticeably.

The chlorophyllide may be conveniently separated from the alkyl chlorophyllides by means of salt formation with ammonia; upon the

[9] Ann. d. Chem. 387: 366. 1912.

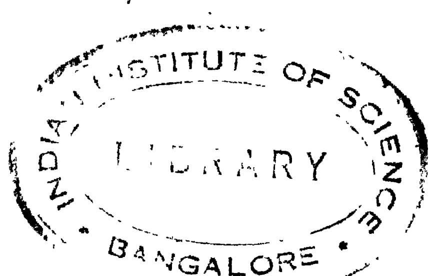
introduction of ammonia gas into a dry ethereal solution the color changes to green, the liquid then becomes turbid and the ammonium salt is precipitated, most conveniently upon talc. If, after filtering off, it is shaken with ether and water, the free chlorophyllide, as a consequence of the dissociation of the salt, passes into the ether. It is undamaged and pure.

The chlorophyllide decomposes when kept for some time, especially in the case where it is subjected to long continued heating in a vacuum as well as when even its dilute solutions stand a long time; it forms magnesium pheophorbide which is insoluble in ether.

Chlorophyllide b, $(C_{32}H_{28}O_2N_4Mg)$ (COOH) $(COOCH_3)$, crystallizes with difficulty even from strongly concentrated solutions. It decomposes easily when its ethereal solution is concentrated. It is obtained from acetone by evaporation or by slow dilution with water in the same crystal form as that of *a*, in glistening hexagonal plates which, under the microscope, appear yellow, yellowish green or olive green. The substance in crystalline condition is stable for a long time, but in an alcoholic solution it allomerizes far more quickly than does its ester.

In alcohols, in which chlorophyllide *b* dissolves very easily, it appears yellow green in thin layers, grass green in thicker ones, and has a brownish red fluorescence. It is somewhat less yellowish tinted in ether, in which it is difficultly soluble; somewhat more so than is *a*. In acetone, *b* dissolves just as easily as does *a*, and in petroleum ether, just as little.

The carboxylic acid *b* is still more strongly acid than chlorophyllide *a*. It may be almost quantitatively extracted from its ethereal solution (0.02 g. in 100 cc.) even by 0.0005 *N* KOH. Chlorophyllide *b* is completely extracted by 0.01 *N* Na_2CO_3 and by 0.01 *N* NH_3. It is abundantly extracted even by 0.001 *N* NH_3. Furthermore, the substance distributes itself between ether and sodium bicarbonate. The alkaline solutions are greenish yellow to yellowish green; those of *a* are green.



XII. THE YELLOW PIGMENTS OF CHLOROPLASTS.

1. Occurrence of the Carotinoids.

Berzelius[1] was the first chemist who sought to isolate a yellow pigment, which he called "leaf-yellow" or "xanthophyll," from autumn leaves by extraction with alcohol. As is well known, large quantities of yellow pigments accompany chlorophyll, even in green leaves. Under the microscope, their beautiful crystals often attracted the attention of botanists, and chemists who occupied themselves with chlorophyll often came upon them in the extracts.

A. Arnaud,[2] during the years 1885–1887, thoroughly investigated a crystalline yellow material, which accompanied chlorophyll. He discovered that this substance was very probably identical with carotin, the pigments of carrots (Daucus carota). A. Hansen also suspected the identity and N. A. Monteverde confirmed it. But we find analyses of carotin from leaves given only by H. Immendorf;[3] if his analyses were correct it would follow that the carotin from leaves is different from that of carrots.

Wackenroder[4] was the first to isolate carotin from carrots; Zeise[5] described it more accurately and advanced the formula C_5H_8. A. Husemann[6] afterwards published an extensive work on carotin and assigned to it the formula, $C_{18}H_{24}O$. Arnaud first established the fact that the pigment of carrots is actually a hydrocarbon. His analysis of carotin and of a carotin iodide lead to the formula, $C_{26}H_{38}$, which remained unquestioned for a long time.

Arnaud noticed only carotin as the accompanying material of chlorophyll and proceeded, in his colorimetric determinations of the

1 Ann. d. Chem. 21: 257. 1837.

2 Compt. rend. 100: 751. 1885; 102: 1119 and 1319. 1886; 104: 1293. 1887; 109: 911. 1889; Bull. soc. chim. 48: 64. 1887.

3 Landwirtschaftl. Jahrbücher 18: 507. 1889.

4 Geigers Magazin f. Pharm. 33: 144. 1831.

5 Ann. d. Chem. 62: 380. 1847.

6 Ann. d. Chem. 117: 200. 1861; Archiv d. Pharm; series II. 129: 30. 1867.

leaf yellow that is contained in green plant parts, on the assumption that only a single yellow substance, which may be extracted with petroleum ether, was to be considered. Judging from the statements regarding their solubility and the appearance of their crystals it is very probable that the erythrophyll of Ch. Bougarel, the chrysophyll of E. Schunck and very likely also the etiolin of N. Pringsheim are identical with carotin.

G. G. Stokes[7] and H. C. Sorby,[8] however, recognized long before this that several yellow pigments were present in addition to chlorophyll; Stokes assumed that there were two, and Sorby that there were three, xanthophylls. J. Borodin[9] then called attention to the fact that several crystallizable, yellow substances of different solubilities occur in leaves and he arranged these accompanying substances of chlorophyll in two groups. To one group, that of the carotins, belong crystals which are easily soluble in benzine and difficultly soluble in alcohol. Representatives of the second group dissolve very slightly in benzine and easily in alcohol. These observations were confirmed and added to by N. A. Monteverde[10] and A. Tschirch[11] and especially by M. Tswett[12] and were supplemented by the spectral analytical investigations of C. A. Schunck.[13]

Tswett[14] proposed grouping the different yellow pigments under the name carotinoids. The existence of several yellow pigments in leaves has often been doubted, however. Immendorf, for example, says "Das Carotin ist der einzige gelbe Bestandteil des normalen Chlorophyllkerns." H. Molisch[15] in his treatise "Die Krystallisation und der Nachweis des Xanthophylls (Carotins) im Blatte" leaves the question open nor is it solved in the detailed work of T. Tammes,[16] "Uber die Verbreitung des Carotins im Pflanzenreich."

[7] Proc. Roy. Soc. 13: 144. 1864.

[8] Quarterly Journ. of Microscopical Science 11: 215. 1871; Quarterly Journ. of Science 8: 64. 1871; Proc. Roy. Soc. 21: 442. 1873.

[9] Melanges biologiques tires du Bull. de l'acad. Imper de St. Petersburg 11: 512. 1883.

[10] Acta Horti Petropolitani 13, No. 9: 148. 1893.

[11] Ber. d. d. bot. Ges. 14: 76. 1896; 22: 414. 1904.

[12] Ber. d. d. bot. Ges. 24: 316 and 384. 1906; Die Chromophylle in der Pflanzen- und Tierwelt, Warsaw, 1910, p. 218.

[13] Proc. Roy. Soc. 63: 389. 1898; 65: 177. 1899; 72: 165. 1904.

[14] Ber. d. d. bot. Ges. 29: 630. 1911.

[15] Ber. d. d. bot. Ges. 14: 18. 1896.

[16] Flora 87: 205. 1900.

A decision was brought about by Willstätter and Mieg[17] as a result of the isolation and analysis of a crystalline representative of each of Borodin's two groups.

Carotin, isolated from dried leaves by means of petroleum ether, was really identical with the pigment of carrots; the formula of Arnaud ought to be changed on the basis of more exact analysis. The new determinations result in the average value, $CH_{1.406}$, that is, the empirical composition of cymol, or still more simply, $(C_5H_7)_x$. Molecular weight determinations by physical methods and chemically; namely, by analysis of the iodine addition products that are poorest in iodine, lead to the formula, $C_{40}H_{56}$, for the hydrocarbon.

In alcoholic extracts of leaves a second yellow pigment predominates. Willstätter and Mieg obtained magnificent crystals of this pigment and designated it xanthophyll. It is, similarly to carotin, strongly unsaturated and auto-oxidizable. Its analysis gave the formula, $C_{40}H_{56}O_2$, which was confirmed by molecular weight determinations and iodide analysis; a surprisingly simple relationship to carotin. Oxygen is probably present in the substance in an ethereal combination.

Xanthophyll and carotin may be quantitatively separated by means of their different distributions between petroleum ether and 90 per cent methyl alcohol; the hydrocarbon, carotin, is soluble in the petroleum hydrocarbons. Xanthophyll, the oxygen compound, passes over into the oxygen-containing solvent.

The yellow pigments are so sensitive toward acids that they do not remain unchanged in the preparation of pheophytin and cannot be obtained here in a crystalline state. But in the saponification of chlorophyll with alkalies the mother liquors form a suitable initial material for their isolation. Also, in the preparation of chlorophyll and crystallized chlorophyllides, the greater portion of the carotinoids is obtained as secondary products, for the recovery of which new methods are given below.

There now remains the question whether these two accurately characterized compounds are the only yellow pigments of the chloroplasts. The large yields of carotin and xanthophyll in pure crystallizations certainly make it improbable that still other carotinoids accompany chlorophyll in land plants.

[17] Paper IV.

M. Tswett, however, has apparently succeeded in still further resolving the yellow leaf pigment by means of chromatographic adsorption analysis (see Chapter VI, section 1). On filtering, for example, a carbon disulphide solution of leaf pigments through a column of calcium carbonate, carotin passes through without its being adsorbed. Besides the two green pigments, four zones of yellow pigments, which Tswett distinguishes as xanthophylls α, α', α'', and β, separate. In the spectrum they show small differences with respect to the position of the absorption bands; unfortunately the single pigments were not isolated or described as such. Xanthophyll β passes down in the chromatogram with the most difficulty; α, α', and α'' compounds may be dissolved out by means of petroleum ether which contains 1 per cent alcohol; β, on the contrary, requires a much greater addition of alcohol.

Tswett[18] considers the xanthophyll of Willstätter and Mieg as an isomorphous mixture of two or three xanthophylls in which α predominates. It is not unlikely that the assumption of the esteemed botanist, like so many of his observations, is correct. If the extraordinary similarity of the xanthophyll from leaves to the xanthophyll, mentioned below, from hen egg yolks is considered—the melting point alone distinguishes them—the possibility that the crystals of the xanthophyll from chloroplasts consist of very similar isomorphic and isomeric bodies, for whose separation we have no method, can not be excluded. It might also be possible, however, that xanthophyll has undergone a change in the chromatographic analysis as a result of oxidation, to which it is especially liable when in an adsorbed condition.

In the quantitative determination of the green and yellow leaf pigments, discussed in Chapter IV, carotin and xanthophyll are examples of the carotinoids that are separated by means of their different distribution between petroleum ether and methyl alcohol, without considering the question, which is of little importance there, of the homogeneity of the xanthophyll that has the formula $C_{40}H_{56}O_2$.

If we pass from the pigments of the chloroplasts to the yellow pigments that are also widely distributed in blossoms, fruits and roots, and to those of animal origin, carotin and xanthophyll are not the only crystallized carotinoids that are encountered. The differentiation into two groups—of a pigment easily soluble in benzine and difficultly soluble in alcohol, and of another of reversed solubility—is quite gener-

[18] In the book cited, page 233.

ally shown and useful; this is true also for the lipochromes of the animal kingdom. It is not sufficient to describe the crystals, on the basis of their observation under the microscope, according to their yellow to red color and their change to blue with concentrated sulphuric acid, but their determination requires their isolation in larger quantities and their more exact characterization and analysis.

Carotin, itself, has been isolated from an animal organ, the corpus luteum of the cow, by H. H. Escher[19] in Willstätter's laboratory. An isomeric hydrocarbon, lycopin, was prepared in the pure state by Willstätter and Escher[20] from the berries of Lycopersicum esculentum and investigated by them.

Also, an isomer of xanthophyll became known in the pure state. Willstätter and Escher[21] obtained lutein from hen egg yolks, thus obtaining for the first time a carotinoid of animal origin, and compared it with the xanthophyll of leaves; they agree in almost all their characteristics, but show a considerable difference in their melting points.

With the physiological significance of these pigments there is associated an interest of purely chemical aspect, especially in the case of another remarkable carotinoid which is a member of the xanthophyll group, fucoxanthin, which Willstätter and Page, in an unpublished work, separated in a pure state from the Pheophyceae. This beautifully crystallized compound corresponds to the formula $C_{40}H_{54}O_6$; it shows the group characteristics of the known carotinoids: in solution it is yellow, without fluorescence, auto-oxidizable, and it gives a blue color with concentrated sulphuric acid. Fucoxanthin is distinguished from the others, especially by the much more strongly basic properties which its ethereally bound oxygen possesses and which may be still more considerably increased by treatment with alkali.

With regard to the natural function of carotinoids, the assumption[22] has been expressed that they play a rôle in oxygen respiration. On the other hand, Th. W. Engelmann, [23] in his investigation "Die Farben bunter Laubblätter und ihre Bedeutung für die Zerlegung der Kohlensäure im Lichte," assumed that carotin, like chlorophyll, is

[19] Zeitschr. f. physiol. Chem. 83: 198. 1913.

[20] Zeitschr. f. physiol. Chem. 64: 47. 1910.

[21] Zeitschr. f. physiol. Chem. 76: 214. 1912.

[22] Arnaud, Compt. rend. 109: 911. 1889; Willstätter and Mieg have, for the time being, chosen this conception.

[23] Botan. Ztg. 45: 393. 1887.

concerned with carbonic acid assimilation. A decision in an experimental way is needed to show whether the yellow pigments play a rôle of their own in the process of assimilation, which rôle is not to be considered a chemical function, or whether the green and the yellow pigments cooperate in the process of assimilation, which their mutual presence appears to indicate.

2. Isolation of Carotin and Xanthophyll.[24]

The two carotinoids are yellow in their solutions and not fluorescent; they are auto-oxidizable, stable towards alkalies, but easily decomposable in acid media.

Although Willstätter and Mieg isolated carotin in small quantities (0.03 g. from 1 kg.) in the pure state by the extraction of dried leaves, the two yellow pigments are obtained as secondary products in working up leaves for chlorophyll and its derivatives and usually from 1 kg. of leaf meal there are obtained 0.15–0.2 g. of carotin and 0.4–0.7 g. of xanthophyll in the form of pure crystals.

For the preparation of pure chlorophyll it is necessary to remove the xanthophyll from its petroleum ether solution by means of aqueous methyl alcohol; by this, however, the quantitative separation of xanthophyll from the petroleum ether soluble carotin is simultaneously accomplished.

Secondary products from the preparation of chlorophyll. In the isolation of chlorophyll (Chapter V, section 2) its solution is, after the transference from acetone to petroleum ether, conveniently freed from xanthophyll (only a little of which has been lost by the washing out of the acetone with water) by washing it three times with 80 per cent methyl alcohol. These extracts are intensively greenish yellow. The pigment is extracted from the methyl alcohol by mixing with ether (4–5 l. in all) and diluting with water. The ethereal solution is shaken with 30–50 cc. of concentrated, methyl alcoholic potash in order to saponify the small amount of accompanying chlorophyll *b*. After the return of the green color the chlorophyll may be completely separated by washing several times with water. Besides, in order to lessen chemical action during its isolation, the xanthophyll may be allowed to crystallize from the solution which still contains a little chlorophyll.

The ether is dried with sodium sulphate, evaporated on a water bath to about 30 cc., and mixed with 200–300 cc. of methyl alcohol.

[24] Unpublished.

The remainder of the ether is now driven off by further concentration and the hot, methyl alcoholic solution is filtered. On cooling, the xanthophyll crystallizes in plates of unusually strong surface luster. If a little water is added, in order to make the separation complete, the xanthophyll forms crystalline aggregates which consist of dendritic forms radially arranged. On standing, the spherical form changes gradually into the usual form of lamellar crystals.

The yield of xanthophyll usually amounts to 0.8 g. (or a little more) from 2 kg. of nettle meal.

The carotin finally remains in the petroleum ether mother liquor from the separated chlorophyll. The filtrate from the chlorophyll is concentrated as much as possible in vacuo at 40° and the oily residue is mixed with 300 cc. of 95 per cent alcohol. The carotin begins to separate immediately in steel blue, lustrous rhombohedra and crystallization becomes complete on standing in the cold. A colorless secondary product is mixed with the crystals; this may be quickly brought into solution by the addition of 200–300 cc. of petroleum ether. The carotin is filtered immediately and washed with a mixture of 2 volumes of petroleum ether and 1 volume of alcohol. The yield is 0.25 g.

Furthermore, a little of the yellow pigments, especially carotin, remains in the leaf substance when plant meal is extracted upon the "Nutsch" with 80 per cent acetone in order to isolate its chlorophyll. In order to completely extract the carotin, more aqueous acetone must be used than is required for the isolation of chlorophyll, or the exhausted meal may be extracted further in a percolator with petroleum ether. From the same charge of 2 kg. of nettle leaves, an additional 0.1 g. of carotin could be obtained in a pure state in this way.

Secondary products of crystallized chlorophyll. The ethyl or methyl chlorophyllide from 2 kg. of dry Heracleum leaves (Chapter IX, sections 1 and 2) is deposited, along with the yellow accompanying substances, from the acetone alcohol solution upon talc which, under the microscope, shows red and yellow crystals in addition to the green ones. When the talc upon the "Nutsch" is washed with petroleum ether and ether, the yellow pigments are completely washed away. The filtrate is strongly shaken with 30 cc. of methyl alcoholic potash and this treatment is repeated in order to separate the green pigments in case it is necessary. It is then gradually diluted with water, while shaking the separatory funnel, till the pure yellow, ethereal layer

separates sharply from the green, alkali salt solution. The latter is extracted a second time with ether. After the yellow solution has been washed with water, it is fractionated with methyl alcohol according to the example that has been given of quantitative separation for the determination of the components (Chapter IV, section 3).

For this purpose, shake twice, with 1 l. of 85 per cent methyl alcohol each time, then four times with the same amount of 90 per cent methyl alcohol and transfer the extracts in pairs to a quantity of ether, which finally amounts to 2.5 l.

The xanthophyll in the ether and the carotin in the petroleum ether are washed with water and strongly concentrated by evaporation, at first under ordinary pressure, and finally under diminished pressure at 40°. The xanthophyll is then precipitated with 1 l. of petroleum ether and filtered upon a "Nutsch" through talc, after it has stood for a short time in an ice box. It is then re-extracted with ether (about 1 l.), reconcentrated and diluted with methyl alcohol in order to evaporate the last traces of ether. In order that the crystallization may be of uniform purity the precipitated portion of the xanthophyll is again brought into solution by again boiling it for a short time with small quantities of methyl alcohol.

After the solution has stood for a few days 1 g. of xanthophyll crystallizes. The mother liquor, when a little water has been added to it, produces a further separation, which, after recrystallization, amounts to 0.3 g.

The carotin (0.3 g.) separated from the concentrated petroleum ether solution (150 cc.), after it had been mixed with 600 cc. of 95 per cent alcohol, in elongated, spear-shaped folia with a dark blue luster.

Secondary products from potassium chlorophyllin. After crude chlorophyll (from 5 kg. of nettle leaves) has been saponified in ether with methyl alcoholic potash (Chapter XVIII) there remains above the precipitated chlorophyllin salt a rather pure yellow solution, which is first washed again with concentrated alcoholic potash and concentrated to 250 cc. It is then mixed, without paying any attention to the separation which begins, with 2 l. of ordinary petroleum ether in order to separate the two yellow pigments, and the resultant xanthophyll precipitate is taken up with talc. It is washed upon the "Nutsch" with low-boiling petroleum ether and the xanthophyll is again extracted from the talc with 1 l. of ether. The ethereal solution, after it has been evaporated to 50 cc., is mixed with 1 l. of methyl alcohol and the ether

is completely driven off by a further brief concentration. On standing in the cold, a yield of 2.2 g. of beautifully crystallized xanthophyll is obtained.

The petroleum ether mother liquor from the xanthophyll-talc slowly deposits a little more xanthophyll when it stands in the cold and this is purified by several washings with 90 per cent methyl alcohol; it is evaporated to a few hundred cubic centimeters so that all the carotin still remains in solution, filtered from the colorless secondary products and diluted with four times its volume of 95 per cent alcohol. The carotin (0.6 g.) then crystallizes nicely in leaflets which have a golden luster and it is freed from colorless impurities by washing it with a petroleum-ether-alcohol mixture (2:1). The mother liquor is still intensively colored; the phytol and other oily materials still hold much carotin in solution.

Carotin from Daucus Carota.[25] In order to obtain carotin from carrots, meal that has been prepared from carrots that have been dehydrated by gentle warming is extracted in a percolator by means of petroleum ether and the percolate is very strongly concentrated under diminished pressure at about 40°. The carotin, which is still mixed with large quantities of colorless secondary products, then crystallizes. It may be purified by fractionally precipitating it a number of times from a carbon disulphide solution with absolute alcohol. Colorless substances first separate; then, pure carotin.

The yield from 5,000 kg. of fresh, that is, 472 kg. of dry carrots, amounted to 125 g. of carotin in the pure state.

Escher isolated the very same carotin from an animal organ, the corpus luteum of the cow, and obtained 0.45 g. of the pure hydrocarbon from 10,000 ovaries.

Willstätter and Escher isolated lycopin, a pigment isomeric with carotin, from the tomato according to the following method:

Pure canned tomatoes of commerce were dehydrated in quantities of about 8 kg. by shaking in powder flasks with 4 l. of 96 per cent alcohol. The coagulated mass was strained and pressed by slight pressure. The shaking with 2–3 l. of alcohol and pressing were repeated and the pulp was then pressed with greater pressure to a crumbly mass, in order that it might be dried completely upon a vapor bath and ground in a powder mill. The tomato meal was exhaustively

25 From the graduate work of H. H. Escher. Zur Kenntnis des Carotins und des Lycopins. Zurich. 1909.

extracted in a percolator with carbon disulphide, and the extract concentrated as far as possible, under diminished pressure toward the end, in a bath at 40°. During this evaporation fine needles of lycopin separate. The deep reddish brown mass was diluted with 3 times its volume of absolute alcohol and, after filtering on a "Nutsch," was washed with petroleum ether.

The crude product of lycopin was purified by precipitation from carbon disulphide solution with absolute alcohol or, better, by recrystallizing from gasoline, (B. P. 50–80°) of which, when boiling hot, 4–5 l. were required for 1 g. of lycopin. When cooled in a freezing mixture a loose, brown powder of prismatic crystals separated from the filtered, gasoline solution.

Small amounts of carotin crystallized from the alcoholic mother liquor from the tomato pigment on long standing.

74 kg. of "Purée di Pomodora Concentrata" yielded 5.6 kg. of dry powder, which produced 11 g. of recrystallized pigment; that is, 0.2 per cent of the dry material.

The pigment of hen eggs, lutein, an isomer of xanthophyll and very similar to it, was prepared by Willstätter and Isler from 6,000 egg yolks. A yield of 4 g. of crude, or about 2.6 g. in a pure state, was obtained.

3. Description.[26]

Carotin: The composition and the molecular weight correspond to the formula $C_{40}H_{56}$; it can take up alcohol of crystallization.

Carotin crystallizes in rhombohedra (Plate III, Fig. 1) and in rhombic, almost quadratic, frequently notched plates (Plate III, Fig. 2) of lively, sometimes coppery, sometimes blue, surface luster. By transmitted light they, and even the thin microscopic laminae, are red.

The melting point, depending somewhat upon the manner of heating, is about 174° (corr.), sintering a little previously.

The hydrocarbon is very difficultly soluble in boiling alcohol and methyl alcohol and almost insoluble in them when cold. About 900 cc. of boiling ether are required for 1 g. Carotin dissolves rather difficultly in low boiling petroleum ether; 1 g. in 1.5 l. in a reflux condenser. It is difficultly soluble in acetone; rather easily in benzol; very easily in chloroform and exceedingly easily in carbon disulphide.

[26] Paper IV and Zeitschr. f. physiolog. Chem. 64: 47. 1910; 76: 214. 1912 and 83: 198. 1913; Vol. 64 of Zeitschr. f. physiolog. Chemie contains colored reproductions of our carotinoid crystals.

Its distribution between petroleum ether and alcohol is characteristic. If a petroleum ether solution is mixed with methyl alcohol that contains very little water the methyl alcoholic layer remains colorless.

The solutions are intensively yellow; concentrated solutions are deep orange in color, but red in carbon disulphide.

Carotin dissolves with an indigo blue color in concentrated sulphuric acid.

Carotin is unsaturated and auto-oxidizable; it bleaches when it stands in the air and, as a result, its weight increases about 35 per cent; in a moist room, about 41 per cent. On bleaching, the crystals retain their sharply defined form.

Carotin unites with iodine to form an iodide, which crystallizes in dark violet, copper lustered prisms of the composition, $C_{40}H_{56}I_2$.

The relations of carotin to cholesterin that are often found mentioned in the literature do not exist in actuality.

Xanthophyll: It inclines to crystallization with ethyl and methyl alcohol; when precipitated with petroleum ether from chloroform, it is free from solvents. Its formula is $C_{40}H_{56}O_2$.

The typical crystal forms are elongated plates (Plate III, Fig. 3) and prisms with swallow tail shaped indentations (Plate III, Fig. 4). The crystals are pleochromatic and show a marked, often steel blue, luster. The crystals are yellow by transmitted light and red only where several crystals cross each other. They are therefore easily distinguished from carotin, although the color in solutions is very similar. Also its behavior toward concentrated sulphuric acid, toward halogens and toward oxygen is identical. An ethereal solution of xanthophyll bleaches on contact with air, even in the dark, very quickly; much more quickly than does carotin.

Xanthophyll, in alcoholic solution, does not give a color change when mixed with somewhat concentrated hydrochloric acid, although Tswett states that his xanthophylls, especially the β form, showed a green and blue color action when their alcoholic solutions were mixed with hydrochloric acid. But xanthophyll is very easily damaged, even more readily so than is carotin, by the action of weaker acids.

The solubility of xanthophyll is essentially different from that of carotin. If an alcoholic solution of xanthophyll is mixed with petroleum ether and the solvents separated by means of a little water, by far the greater part of the pigment is found in the alcoholic layer.

Xanthophyll is so insoluble in petroleum ether that the solvent is not even colored. It dissolves rather difficultly in methyl alcohol (1 g. in 700 cc. of boiling, or in 5 l. of cold, methyl alcohol) but still much more easily than does carotin. It is considerably more easily soluble in ethyl alcohol, rather easily soluble in ether (1 g. in 300 cc. on boiling), very easily in chloroform, and quite difficultly soluble in carbon disulphide.

Melting point 173–174° (corr.).

Xanthophyll might contain oxygen in ethereal combination for it gives neither carbonyl, alcohol, nor acid reactions. But it appears to form a very easily dissociated addition product when its ethereal solution is mixed with concentrated methyl alcoholic potash. Much of the xanthophyll passes into the alkali and it is only slowly given up when extracted with ether, but immediately so upon the addition of water. No alteration of the xanthophyll occurs in connection with this.

Comparison of color intensities.[27] Carotin and xanthophyll are very different in their color intensities but there is no simple ratio of their color strengths. This varies with the solvent and with the concentration. Carotin is always stronger in color. The diluted solutions of both are not comparable because they (in carbon disulphide as well as in ether) are different in hue: carotin is more red; xanthophyll, rather greenish tinted.

The purity of the preparations that were used for comparison was confirmed by elementary analysis (carotin, found according to Pregl's method, C. 89.4, H. 10.6; xanthophyll, C. 84.2, H. 10.1).

10^{-6} Mole in 220 cc. of Carbon Disulphide.

Depth of layer of Carotin in mm.	Depth of layer of Xanthophyll	Intensity ratio
12.0	50	4.1
25.5	87	3.4
38.5	120	3.1
85.0	180	2.1

10^{-6} Mole Carotin in 200 cc. of Petroleum Ether-Ether.
Xanthophyll in Ether.

Depth of layer of Carotin in mm.	Depth of layer of Xanthophyll	Intensity ratio
10.0	20	2.0
40.0	60	1.5
91.0	120	1.3

[27] Unpublished.

The color of the yellow pigments appears more intense in carbon disulphide than in the other solvents; in the case of carotin the ratio is difficult to determine, because even a thin layer of the carbon disulphide solution is red; in the case of xanthophyll the carbon disulphide solution is five times more intense than the ether solution.

Absorption spectra. In the spectroscope the alcoholic solutions of carotin and xanthophyll show a spectrum that consists of two bands in the blue and indigo blue in addition to the end absorption which starts at almost the beginning of the violet.

A spectrographic photograph (Chapter XXV, Plate XI) discloses in this end absorption, in the region of the extreme violet which already

Table for the Comparison of Carotin and Xanthophyll.

	$C_{40}H_{56}$	$C_{40}H_{56}O_2$
Typical crystal form	rhombic plates	swallow tail shaped prisms
Color by transmitted light	red	yellow
Passes, on the separation of petroleum ether and methyl alcohol,	into the upper layer	into the lower layer
In petroleum ether	quite soluble	insoluble
In alcohol	very difficultly soluble	quite soluble
In carbon disulphide	exceedingly soluble	rather difficulty soluble

appears dark, a clearly defined, third band (for carotin at $\lambda = 425\ \mu\mu$, for xanthophyll at $\lambda = 420\ \mu\mu$.

The bands, in the case of xanthophyll as contrasted with carotin, are displaced slightly toward the violet.

In the leaf extract the second and third bands of the carotinoids are masked by the very intensive absorption of the chlorophyll components in the violet. The first absorption band of the yellow leaf pigments falls in the gap between bands V and VI of chlorophyll *a*, but it is almost coincident with the band VIII of a not too thin layer of chlorophyll *b*.

The light absorption of the carotinoids is, therefore, not complementary to that of the chlorophyll components.

In carbon disulphide the difference between carotin and xanthophyll is greater. The absorption bands are here strongly displaced toward the red end of the spectrum. Although carotin shows, in the

visible region, only one band in the green and one in the blue, there is, in the case of xanthophyll, an additional third distinct band in the indigo blue.

Measurements with a grating spectroscope:

0.005 g. in 1 l. of alcohol (Plate XI).

	Carotin		*Xanthophyll*	
Thickness of layer in mm.	5	10	5	10
Band I	492——478	492—476	484——472	488—471
Band II	459——446	459—445	454——441	454—440
End absorption[28]	415—	419—	419—	420—

0.005 g. in 1 l. of carbon disulphide (Plate XI).

	Carotin		*Xanthophyll*	
Thickness of layer in mm.	10	20	10	20
Band I	524 . . . 510	525——508	515 . . . 501	516——501
Band II	489—475	490—474	482—469	483—467
Band III	—	—	—	447 . 441

Lycopin: The isomer of carotin forms a carmine red, velvety lustered aggregate of elongated prisms, which are cleft at their extremities or of needles. Under the microscope the crystals are brownish red to carmine red; melting point 168–169° (corr.). Its solution in ether stains the glass wall much less than does the carotin solution. Its hot, saturated alcoholic solution is dark yellow; its solution in carbon disulphide is a beautiful, bluish tinged red.

Lycopin is more difficultly soluble in alcohol and other solvents than is carotin; 1 g. requires 3 l. of boiling ether, 10–12 l. of boiling petroleum ether, or 50 cc. of carbon disulphide at ordinary temperature. In its distribution between alcohol and petroleum ether lycopin behaves as does carotin.

In its reactions, lycopin is similar to carotin; it takes up oxygen with even more avidity.

The absorption spectrum in carbon disulphide shows two bands in the green and one in the blue.

Lutein. On comparison with xanthophyll only one distinct difference has been observed between these isomers; it melts at 195–196° (corr.).

[28] The real beginning of the end absorption, *i.e.*, the limit of the third band in the violet, we determined by means of a spectrographic photograph of a layer of carotin 10 mm. thick to be at $\lambda = 430\ \mu\mu$, and for xanthophyll, at $\lambda = 425\ \mu\mu$.

4. The Preparation and the Description of Fucoxanthin.[29]

In Chapter V (section 4) there has been described the extraction of the Pheophyceae on a large scale and their working up for chlorophyll. The filtrate from the crude chlorophyll-talc contains all the fucoxanthin together with a large portion of the xanthophyll and traces of chlorophyll. The isolation of the fucoxanthin[30] is carried out according to the method that served for the quantitative determination of the Pheophyceae pigments. The fucoxanthin is separated from the xanthophyll on the basis of its somewhat different distribution between aqueous methyl alcohol and ether-petroleum ether and, at the same time, it is also separated from most of the colorless accompanying substances which make its precipitation difficult. They remain, for the most part, in the ethereal layer.

The brown algae were usually worked up in quantities of from 15–20 kg. The combined fucoxanthin-containing filtrates from the crude chlorophyll (40 l. from 20 kg.) are introduced, each 4 l. portion, into 1 l. of a mixture of petroleum ether (3 vol.) and ether (1 vol.), and mixed with 1½ liters of water. After thorough shaking, the aqueous acetone layer is only pale yellowish green. The deep orange yellow solutions that are obtained are freed from acetone by very careful washing, whereby troublesome emulsions occur altogether too easily, and are evaporated in vacuo at ordinary temperature to a total volume of 500 cc. If floccules of fucoxanthin precipitate during this operation dilute again with 0.5 l. of ether. The separation of the two yellow pigments is now undertaken by shaking carefully about four times, each time with 1 l. of 70 per cent methyl alcohol that has been saturated with petroleum ether, and extracting twice more with half of that amount. The xanthophyll that has been carried along is removed from the dark brown, orange tinged, methyl alcoholic solution by shaking it once with an equal quantity of a mixture of 5 volumes of petroleum ether and 1 volume of ether. Since this wash fluid takes up a not insignificant portion of fucoxanthin, it is evaporated in a vacuum to a few hundred cubic centimeters, diluted with an equal quantity of ether, and extracted twice more with 70 per cent methyl alcohol. These last extracts are, of course, also washed with ether-petroleum ether.

[29] From the unpublished investigations of R. Willstätter and H. J. Page.

[30] For the history of fucoxanthin, whose solutions have been described by M. Tswett as well as by H. Kylin, see Chapter IV, section 5.

The fucoxanthin in all the methyl alcoholic solutions is now transferred, in portions, to considerable ether by careful fractional separations, and the filtered solution is concentrated at a low temperature to about 200 cc.; that is, almost to a sirupy consistency. Upon the addition of low boiling point petroleum ether (at most, 1 l.) the fucoxanthin precipitates in brick-red floccules which are already quite pure. The yield of such crude product amounted to 2 g.

Reagents that contain mineral constituents (for example, well water) were avoided in this process of isolation; also, no drying was undertaken with calcium chloride. In some of the first experiments for the preparation of fucoxanthin, Willstätter and Forsén did not exercise this precaution and arrived at peculiar observations, having obtained fucoxanthin preparations which, after single and after repeated recrystallizations from methyl alcohol, formed pure, beautiful prisms with a melting point of 145–150° and which left, when ashed, 3–4 per cent of pure CaO.

Pure fucoxanthin, on the contrary, is ashless. Its composition is expressed by the formula, $C_{40}H_{54}O_6$.

The crude product is very easily soluble in all the organic solvents except methyl alcohol and petroleum ether. It recrystallizes well from a small amount of methyl alcohol and is obtained in bluish-lustered, brownish red, long prisms of monoclinic habit (Plate III, Fig. 5). Under the microscope the crystals are amber yellow and, where several prisms cross, brown. Its powder is brick red.

1.66 g. of the recrystallized substance dissolves in 100 g. of boiling methyl alcohol; at 0°, 0.41 g.; the crude product is much more soluble. The crystals dissolve with some difficulty in ether, rather easily in carbon disulphide, and easily in ethyl alcohol.

Fucoxanthin appears in a second, very characteristic, crystalline form; that is, in large, regular, hexagonal plates (Plate III, Fig. 6), if its alcoholic solution is placed over water with the exclusion of air. They are red; viewed through the microscope by transmitted light they are pure yellow to red, depending upon their thickness.

The melting point of fucoxanthin lies between 159.5 and 160.5° (corr.).

The ethereal solution is orange yellow, tinted pure yellow; the alcoholic solution is somewhat rusty colored and tinged a brownish yellow; fucoxanthin is much redder in carbon disulphide.

The pigment does not exhibit fluorescence.

The solid material does not absorb any oxygen; after drying it undergoes, in the air, a 7 per cent increase in weight and then shows very great fluctuations in weight, depending upon the humidity of the atmosphere. The preparation subsequently returns to its original weight when placed in a desiccator. Solutions of fucoxanthin, on the other hand, spoil easily. They appear to be sensitive to oxygen, especially in the light.

Iodine unites instantaneously with fucoxanthin and a crystallized iodide may be obtained from an ethereal solution.

Characteristic of the pigment are its basic properties. While carotin and xanthophyll give the well known blue color phenomenon with concentrated sulphuric acid only, the ethereal solution of fucoxanthin reacts with hydrochloric acid exactly like a weak nitrogenous base. The ethereal solution is immediately bleached by 30 per cent hydrochloric acid and the acid layer shows a splendid, blue violet color which, on great dilution, is sky blue. This color is due to a dye salt, probably an oxonium salt. Even 25 per cent hydrochloric acid is thus colored, though only feebly. The dye salt is dissolved by the aqueous hydrochloric acid only in consequence of its ether content. If the material is isolated from the acid, it is altered and will react partially even with dilute acid.

Fucoxanthin in anhydrous ether gives, with chlorine water, a flocculent precipitate of its hydrochloride.

Its behavior towards alkalies is also very noteworthy. Fucoxanthin does not show any acid properties; it will not pass from ether into aqueous lye at all. On the other hand, it reacts with concentrated alcoholic alkalies to form an easily dissociated addition product, an alteration of the fucoxanthin taking place at the same time. In this behavior it reminds one of pyrone, which, according to R. Willstätter and R. Pummerer,[31] is split up by alkalies as well as by alcoholates, even at room temperature.

The solid material dissolves much more easily in methyl alcoholic potash than in methyl alcohol. A large part of the fucoxanthin passes from ether into concentrated methyl-alcoholic potash and it is given up from this to ether only quite slowly; immediately and completely, however, upon the addition of water. The pigment in the thus ob-

[31] Ber. d. d. Chem. Ges. 37: 3740, 1904. and 38: 1461. 1905.

tained ethereal solution, which is also yellow, possesses markedly strong basic properties; much stronger than those of undamaged fucoxanthin. The solution, in fact, reacts even with 0.001 per cent hydrochloric acid. It gives up a large portion of the pigment to 1 per cent hydrochloric acid and almost all the pigment, though with the separation of floccules, to 3 per cent hydrochloric acid.

Absorption spectrum, 0.005 g. fucoxanthin in 1 l. alcohol.

Thickness of layer in mm.	5	10
Band I	486—469	493—469— —
Band II	455—440	} 454—
End absorption	440—	

XIII. PHEOPHYTIN.[1]

1. Definition.

According to Willstätter and Hocheder (1907) pheophytin is formed from chlorophyll by carefully treating it with oxalic acid. It is a mixture of the two phytyl pheophorbides *a* and *b*. The action of acids upon chlorophyll and upon the phyllins consists in the complexly bound magnesium being split off quantitatively; the magnesium is simply replaced by hydrogen in this action. The products that arise are free from mineral constituents. The two ester groups in the chlorophyll remain intact.

The color changes from green to brown; the solubility is greatly decreased so that it is possible to separate nearly the whole chlorophyll content of the extract in the form of the magnesium-free derivatives; susceptibility to decomposition becomes much less, and its sensitiveness in alcoholic solution (allomerization), especially, is eliminated.

The possibility of altering chlorophyll by means of strong acids had been known for decades. But success had not been attained in isolating a pure product of the acid cleavage and thus obtaining phytol, which constitutes a third of the chlorophyll molecule. The action of acid upon the leaf extract had mostly been so manipulated that the chlorophyll was ruined in the process.

The formation of pheophytin was, however, not conditioned by mild acid cleavage alone, but depended especially upon the use of good chlorophyll solutions. Every advance in the preparation of leaf extracts meant at the same time an improvement in the preparation of pheophytin.

The pheophytin that is formed by the treatment of crude chlorophyll with acid is suitable for the further systematic decomposition of chlorophyll and is the best initial material for the preparation of phytol.

[1] Papers III and VII.

2. The Older Methods for Acid Cleavage of Chlorophyll.[2]

E. Frémy[3] began the investigation of the action of acids upon chlorophyll and introduced the names, phylloxanthin and phyllocyanin, whose meanings have subsequently changed considerably.

Frémy allowed strong hydrochloric acid and ether to act upon the yellow precipitate that is formed from the alcoholic extract by means of aluminum hydroxide and alkaline liquor, or directly upon the residue from the evaporation of the extract. After separation the ether was pure yellow and the acid blue. Frémy assumed that two components of chlorophyll were present in these layers and he called the yellow one phylloxanthin, the blue one phyllocyanin. Frémy then heated alcoholic extracts with barium hydroxide and liberated the so-called phyllocyanic acid from its barium salt by means of sulphuric acid. He imagined that chlorophyll was a colored fat, in which the indifferent phylloxanthin played the role of glycerin, and phyllocyanin, that of the fatty acid. Later, Frémy preferred the hypothesis that chlorophyll was a mixture of phylloxanthin and the potash salt of phyllocyanic acid.

Originally, therefore, phylloxanthin consisted of a naturally quite impure mixture of yellow accompanying substances that had been altered by acids, and phyllocyanin, of a mixture which varied, according to the manner of production, of the different cleavage products produced by the action of acids upon the two chlorophyll components.

With the same names E. Schunck[4] designated two products which he had prepared in his investigations during the years 1885–1896 by means of the energetic action of hydrochloric acid upon chlorophyll without, however, publishing their analyses.

Fresh grass was extracted with boiling, strong alcohol and the filtered solution was treated with a stream of hydrochloric acid gas. A dark mass separated which, on treatment with ether and concentrated hydrochloric acid, furnished two portions; the portion that was formed in much larger quantity and which did not go into strong hydrochloric acid was phylloxanthin; the portion that was taken up by the acid and which was repeatedly recrystallized from acetic acid was phyllocyanin.

Phyllocyanin is a crystalline compound which, on solution in ether, is dark or olive green.

[2] Paper XX.

[3] Compt. rend. 50: 405. 1860; 61: 188. 1865; 84: 983. 1877.

[4] Proc. Roy. Soc. 38: 336. 1885; 39: 348. 1885; 42: 184. 1886; 44: 48. 1888; 50: 302. 1891; 55: 351. 1894.

Phylloxanthin is very similar to phyllocyanin, but it contains fat; it yields an ash which always shows an integrant content of iron. It is yellowish brown in solution. If its ethereal solution is shaken with concentrated hydrochloric acid the latter remains colorless.

With regard to the relationship between the two, E. Schunck and L. Marchlewski[5] came to the conclusion that phylloxanthin was an intermediate product in the formation of phyllocyanin.

M. Tswett[6] tested the formation of phylloxanthin and phyllocyanin under the experimental conditions given by E. Schunck and traced the two cleavage products, in conformity with an old supposition of G. G. Stokes,[7] back to the two components of chlorophyll. By the aid of his chromatographic method he showed that phylloxanthin is essentially the first cleavage product of chlorophyll *b*, and that phyllocyanin is a secondary cleavage product of the *a* chlorophyll component. In order to understand these observations there was lacking only knowledge regarding the saponifiable groups contained in the chlorophyll and consideration of allomerization, as a result of which all the chlorophyll derivatives mentioned in the earlier literature had been spoiled.

A product much more closely related to chlorophyll is chlorophyllan, which F. Hoppe-Seyler[8] obtained in the year 1879 by extracting grass with boiling alcohol; it was isolated from these extracts, which had been previously concentrated by evaporation, by a series of separations and purifying operations. Although crystallizable, it was not a pure compound. It gave considerable ash which contained 1.38 per cent of phosphorus and 0.34 per cent of magnesium, and it also disclosed a glycerin and a cholin content. Its composition, therefore, led Hoppe-Seyler to the supposition that chlorophyll belongs to the lecithins.

About the same time, A. Gautier[9] obtained a similar preparation and described it as crystallized chlorophyll.

The formation of chlorophyllan, whose color in solutions is olive green, depends, according to Willstätter and Hug, upon the unex-

[5] Ann. d. Chem. 278: 329. 1894; 284: 81. 1894; Proc. Roy. Soc. 57: 314. 1895; Marchlewski, L. Die Chemie des Chlorophylls (1895), p. 63; Roscoe-Schorlemmer VIII, 889; Biochem. Zeitschr. 3: 303. 1907.

[6] Bioch. Zeitschr. 5: 6. 1907; 6: 373. 1907; 10: 404. 1908.

[7] Proc. Roy. Soc. 13: 145. 1864; see in addition Proc. Roy. Soc. 50: 311. 1891.

[8] Zeitschr. f. physiol. Chem. 3: 339. 1879; 4: 193. 1880; 5: 75. 1881.

[9] Compt. rend. 89: 861. 1879.

pected decomposition by plant acids of chlorophyll that has already been partially allomerized by its treatment with solvents. Chlorophyllan can be obtained only with an ash content and as a mixture with colorless material (fat, lecithin, among others) by Hoppe-Seyler's method.

3. Preparation of Pheophytin.

By the method of Willstätter and Hocheder.[10] The meal of dried, stinging nettle leaves or grass was extracted in a flask with 96 per cent alcohol (1.5 to 2 l. per kg.) in the cold; after filtering by suction and washing, the extract served for the extraction of another similar charge.

The double extract that is thus obtained (Chapter III, section 2a) shows, as a result of the action of acid, a beautiful change of color to dark brown and, at the same time, the strong fluorescence disappears.

The reaction was effected by the addition of a concentrated solution of oxalic acid (containing water of crystallization) in 96 per cent alcohol, which had been freshly prepared in the cold. As a rule, 2.5–5.0 g. of oxalic acid were required for a liter of the double extract. The chlorophyll solution was then treated, all at once, with 2.5 g. of oxalic acid per kilogram of plant material and, in case a total change of color did not then take place in the course of 15 to 30 minutes, the quantity of acid still needed to complete the change of color was added in small portions at intervals.

The settling of a flocculent precipitate, consisting chiefly of pheophytin and salts of oxalic acid, especially those of magnesium and calcium as well as those of potassium and aluminum, begins to take place even during the addition of the oxalic acid. The separation was complete after standing a day and the precipitate settled to the bottom so compactly that the greater portion of the liquor could be decanted. The mother liquor still contains some pheophytin and very much of the yellow materials that accompany chlorophyll, but they can not be obtained from it in a crystalline state.

The precipitated mixture of pheophytin and oxalates was filtered upon a suction filter, washed several times with alcohol and dried in a vacuum desiccator. A single reprecipitation from a chloroform solution by means of alcohol always served for the removal of the salts and as the first purification; the metallic compounds were left behind on

[10] Ann. d. Chem. 354: 218. 1907.

solution and the mother liquor retained the organic impurities. Only in those cases where the preparation of the pheophytin was on a very large scale did it prove profitable to make a second separation from chloroform by means of alcohol.

The filtration of the chloroform solution is difficult and requires a rather large dilution. It was first filtered on a large suction filter, using a pump, but since some of the fine precipitate passed through, it was necessary to filter again three or four times through hardened filters in order to obtain the pheophytin free from ash. The filtrate was strongly concentrated under reduced pressure at ordinary temperature and the thick, almost black, solution was precipitated by means of five to ten times its volume of 96 per cent alcohol.

Working with many plants, the yield of pheophytin usually amounted to 3 g. from 1 kg. of dry plant material; in the case of leaves that were rich in chlorophyll considerably more was obtained; for example, 100 kg. of stinging nettle produced (without using the mother liquor) 424 g. of pheophytin.

A modification of this method consisted in the preparation of crude chlorophyll solutions by the use of percolators (Chapter III, section 2a); at first the leaf meals were macerated in the percolators 24–48 hours and subsequently percolated. The "precipitation coefficient"; that is, the quotient

$$\frac{\text{chlorophyll precipitated as pheophytin}}{\text{dissolved chlorophyll}},$$

proved to be more favorable with quick percolates which had been produced without any maceration at all. Thus we obtained from 44 kg. of stinging nettle meal, with which four percolators were charged, 208 g. of pure pheophytin; that is, 4.5 g. from 1 kg.

The new method.[11]

According to our investigations for the comparison of methods of extraction the best method is the quick extraction of the leaf meal upon the suction filter by means of aqueous solvents:

1. Acetone with 10–20 per cent (by volume) of water.
2. Alcohol with 10 per cent (by volume) of water.

In this procedure a thin layer of plant material is used so that the chlorophyll cannot reprecipitate farther down in the leaf meal from the concentrated solution that is first formed.

[11] Unpublished.

Extracts so obtained make possible great improvements in the preparation of pheophytin.

The pheophytin of Willstätter and Hocheder was a pure chlorophyll substance; that is, free from fat, wax and other colorless or colored admixtures. Still, the chlorophyll in the extracts that were employed was in many cases altered;[12] it was allomerized during the elaboration of certain plant meals, stinging nettle especially. Consequently many pheophytin preparations, on cleavage, produced not only the normal derivatives, phytochlorin *e* and phytorhodin *g*, but in addition, the feebly basic compounds, chlorin and rhodin. Furthermore, the phytol ester group was exposed to alcoholysis during the rather long contact of the extract with the leaf meal. Consequently, the composition of the pheophytin was not constant.

The extracts that are obtained by the new rapid method with the use of aqueous solvents contain chlorophyll in an unchanged state and consequently produce a pheophytin that consists of a pure mixture of phytyl pheophorbides *a* and *b* in the same ratio as they occur in nature and which gives, upon saponification, the two normal cleavage products only. A further advance consists in the fact that these new extracts produce immediately, upon acidification with hydrochloric acid, pure pheophytin which requires no reprecipitation or reseparation. It would have been impossible by acidifying with hydrochloric acid (instead of oxalic acid) to precipitate pure pheophytin from the extracts that were obtained by the older methods; reprecipitation was absolutely essential.

The acetone and alcohol extracts that are obtained by the new method may serve for the preparation of pheophytin.

The largest yields of chlorophyll are obtained by the extraction with acetone (95 per cent) but this gives, on direct acidification, very impure pheophytin. The acetone extracts should consequently be elaborated for pheophytin by means of a precipitation of the crude chlorophyll upon talc by their dilution with water (Chapter V, section 3), re-extraction of the chlorophyll from the talc with 92 (not higher) per cent alcohol and acidification.

Alcoholic extracts are more suited for the preparation of pheophytin by direct acidification.

The following example shows the effect upon the yield and the purity of the pheophytin of the addition of varying amounts of water to the alcohol that has been used.

[12] Paper XIV.

Each kilogram of commercial stinging nettle meal (chlorophyll content, 5.1 g.) was extracted by the suction filter method with 95, 90 and 85 per cent (by volume) alcohol under exactly similar conditions (according to the method of Chapter III, section 2) and the extracts (in every case, 0.9 l.) were precipitated with hydrochloric acid.

From this comparison the following method is obtained for the preparation of pheophytin.

The initial material, which was available on a large scale, was the medium finely ground stinging nettle leaves of the wholesale drug trade ("Techn. Brennesseln"), the chlorophyll content of which was 1/2–2/3 per cent. Our shallow, stoneware suction filters of 50 cm. diameter were each filled under the suction of a vacuum pump with 4 kg. of dry leaf meal. This formed a layer that was only 4–5 cm. thick. Without any further use of suction 2 l. of 90 per cent alcohol are allowed to soak into the meal, the vacuum cock is then opened and

Alcohol	95%	90%	85%
Chlorophyll content	4.28 g.	5.04 g.	4.67 g.
Pheophytin yield	3.15 "	3.70 "	3.20 "
Purity of the pheophytin	90%	100%	100%

4 more l. of solvent are poured on, in several portions. The meal is completely extracted in 20 minutes. In order to free the filtrate (4. l.) from fine particles of meal, it is filtered upon a small "Nutsch" through several layers of filter paper.

The extracts produced by adjacent "Nutschen" are united in pairs and immediately mixed with 160 cc. of 10 per cent alcoholic hydrochloric acid. The change of color follows quickly and, at the same time, there begins a separation of the pheophytin in the form of fine grains. This is complete in an hour. After this has elapsed the clear, brownish yellow mother liquor, in which on more prolonged standing only a little, less pure precipitate forms, is carefully decanted from the compact precipitate, filtered upon a suction filter with the use of strong suction and washed (while preventing the formation of fissures) three times, using 100 cc. of 96 per cent alcohol each time. The precipitate is subjected to pressure and then, while still moist with alcohol, it may be very easily comminuted. When dry, however, this may be done with extreme difficulty only. It is, therefore, while still moist,

cut with sharp, silver spatulas into small pieces that are suitable for further use, particularly for saponification with alcoholic potash, and dried in large, stoneware, vacuum desiccators containing perforated plates of the same material.

A precipitation coefficient of 0.75 to 0.85 was obtained and a yield of 3.6 to 5.0 g. of pheophytin, which required no further purification, was secured from 1 kg. of leaf meal.

A single laboratory worker can handle 40–48 kg. in a day, with a yield of 180–250 g. of pheophytin; the mother liquor, on rectification, gives an 80 per cent recovery of 93 per cent alcohol (by volume).

Considerably better yields of pheophytin were obtained by the elaboration of stinging nettle leaves with a good chlorophyll content (which we collected ourselves) than by the use of commercial stinging nettle leaves.

It is advantageous to fill the large suction filter each time with only 2 kg. of the material that is richer in chlorophyll and to use double the amount, *i.e.,* 6 l., of 90 per cent alcohol for the extraction. The extract (4.4 l.) was mixed with more hydrochloric acid, corresponding to its greater volume; for example, with 100 cc. of 15 per cent alcoholic acid, and filtered after only one hour.

A yield of 12.9 g. of very pure pheophytin was obtained and, as the following numbers show, this was a very good proportion of the chlorophyll content of the stinging nettle.

Chlorophyll in 1 kg. of stinging nettle: 8.6 g.;
Chlorophyll in the extract from 1 kg. of stinging nettle: 7.75 g.; extraction coefficient[13] = 0.90;
Pheophytin from 1 kg. of stinging nettle: 6.45 g.; precipitation coefficient = 0.83;
Yield = $0.90 \times 0.83 = 75$ per cent of the theoretical yield.

We have worked up still better crops of stinging nettle leaves, which had a chlorophyll content of 10 g. per kilogram, for other purposes. These would have made possible a yield of 8 g. of pheophytin.

4. Description.[14]

Pheophytin is obtained in an ash-free condition; preparations on the largest scale gave 0.04 per cent; on a small scale, still less ash.

[13] The quotient of the extracted and the extractable pigment can be thus designated.

[14] Paper III; partly unpublished.

The purity of a preparation is tested (Chapter IV, section 1b) by colorimetric comparison with a mixture of methyl pheophorbides *a* and *b* of corresponding component ratio. According to their color value preparations prepared on a large scale, without purifying, should be between 98 and 100 per cent pure. They consequently do not contain any colorless admixtures.

Yellow accompanying materials, even in traces, are betrayed by treatment of the ethereal solution with methyl alcoholic potash; the ether should assume only a very slight yellow color or none at all.

The phase test is carried out in the same manner; the phase is brown and soon changes into olive green to green, according to the concentration of the alkali.

In the cleavage test, which is carried out by the introduction of a concentrated pyridine solution of pheophytin into boiling methyl alcoholic potash and then boiling for half a minute, there are formed only the two normal derivatives, phytochlorin *e* and phytorhodin *g*, whose ratio is usually about 2.5 : 1.

It was difficult to obtain such a result without any hitch, particularly in the elaboration of the ordinary initial material, dry stinging nettle, whose extracts are exceedingly prone to the formation of weakly basic compounds. A perfectly pure mixture of chlorophyll derivatives is obtained only if and when

1. the plants are quickly extracted;
2. the extract is immediately acidified;
3. the cleavage is carried out under exactly determined conditions (Chapter IV, section 2).

Determination of the phytol number also shows that the easily attacked ester group is undamaged. An admixture of phytol-free pheophorbide may be detected with much more ease and greater sensitiveness by the basicity test; namely, by shaking an ethereal solution with 22 per cent hydrochloric acid. This does not extract the phytol ester and is, therefore, but very slightly colored. Hydrolyzed and alcoholyzed pheophytin, which in the old, slow extraction methods (double extractions, percolates) was an unavoidable admixture, is easily extracted by this acid.

Pheophytin, on the contrary, is so feebly basic that it just begins to color 25 per cent hydrochloric acid; 28–29 per cent acid extracts the pure component *a*. Phytol is split off hydrolytically in the strong acid, more quickly so in very concentrated acid.

Phytyl pheophorbide does not possess any acid properties.

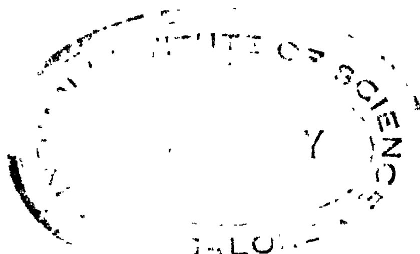
Pheophytin combines very easily with metallic salts to form intensively colored, very stable, complex compounds. Contact with base metals must, therefore, be avoided in its preparation. With ferric salts in the cold there is formed at once a beautiful, greenish tinted, blue solution, which fluoresces very weakly. Zinc acetate gives a beautiful, blue green solution, which is red by transmitted light and distinguished by a strong fluorescence. Copper acetate, even when greatly diluted, converts the brown color of pheophytin into an intensive green; the solution does not fluoresce.

Pheophytin is a wax; it cannot be obtained in a definite crystalline form, but forms arborescent, crystalline structures. It is bluish black in color; olive brown in solution, and red by transmitted light when in layers of great thickness. It is rather difficultly soluble in hot alcohol, very difficultly soluble in cold alcohol, and may be separated[15] from it by recrystallization. It dissolves slowly, but considerably, in ether; very easily in benzol; extremely easily in chloroform, but it is almost insoluble in petroleum ether.

Pheophytin decomposes when it is heated with concentrated nitric acid; a nitrogenous derivative of phytol then floats upon the clear fluid as a colorless layer of oil.

The reaction of an ethereal pheophytin solution with concentrated nitric acid is also characteristic: the ether turns blue upon shaking, while no pigment is taken up by the acid; when washed with water the ethereal solution again assumes the original olive color.

[15] Ann. d. Chem. 354: 221. 1907.



XIV. METHOD OF SEPARATION AND DETERMINATION OF THE CHLOROPHYLL DERIVATIVES.[1]

1. Fractionation with Hydrochloric Acid.

Since chlorophyll consists of two components and since each of these components contains easily alterable lactam groups, mixtures of cleavage products are consequently formed by its decomposition. No methods existed for the separation and purification of these and their identification was, at first, very uncertain.

These substances of high molecular weight have often the same, often a very similar, composition, and show extensive agreement in their reactions and color phenomena as well as in their behavior toward solvents. As long as such reaction-products occur as mixtures their crystallization is rendered difficult. The usual manner of investigating such chlorophyll derivatives, and in the hands of many investigators actually the only method, was that of spectral analysis. This, indeed, consisted of the determination of absorption maxima only; complete observation of the absorption spectra was not made, for this was much too difficult. The method, as applied, did not prevent the commission of most serious errors. As long as the constants of the pure substances are unknown the method gives little information as to whether the compounds are homogeneous and pure or whether they occur as mixtures. Although the spectral analytical method gives extremely good disclosure of many alterations and decompositions of the pure substances, it is wholly inapplicable to the more complicated mixtures of compounds.

Very many of the decomposition products of chlorophyll undergo a fundamental change of color; that is, of their absorption spectra, upon the evaporation of their ethereal solutions or upon recrystallization or drying in a desiccator and, indeed, this often occurs as a conse-

[1] Partially according to paper I.

quence of but very trifling changes in their molecules such as, for example, the splitting off of water. Thus, the solutions of glaucophyllin, which are a beautiful blue when fresh, become, when evaporated and again taken up by ether, more and more like rhodophyllin; that is, red. Spectroscopically this effect is much greater than, say, that of contamination by rhodophyllin. In the identification of the chlorophyll derivatives by their absorption spectra the condition of the preparation should, therefore, be much more carefully considered than has been indicated heretofore by the literature on the subject.

Consequently, in spite of the importance of the absorption spectra for the description of chlorophyll derivatives, the spectroscopical method was only rarely of value for chemical investigation or for that work which had for its object the isolation of the pigment components; this estimate of the value of the method, which had already been expressed in the first paper on the subject, has often been confirmed since.

Willstätter, jointly with W. Mieg, began the investigation of chlorophyll by means of a method of separation and investigation that was independent of spectroscopical analysis and which was based upon the characteristic, basic nature of all magnesium-free, chlorophyll derivatives.

Chlorophyll itself is neither base nor acid. The decomposition products that are formed with alkalies, the phyllins, have only acid properties; as long as the molecule contains complexly bound magnesium, acid reagents can not come in contact with it without effecting decomposition.

The porphyrins are formed by removing the magnesium from these phyllins. They are compounds of a red color which have both acid and basic reaction and are employed in the method about to be described.

The compounds still more closely related to chlorophyll, pheophytin and the other pheophorbides, are distinctly more weakly basic than the porphyrins. They therefore require for solution strong hydrochloric acid and, in these solutions, are very easily subject to changes so that the hydrochloric acid method of Willstätter and Mieg was not originally applicable to them. Only after these saponifications of definite ester groups had been exactly defined in recent years were

the changes avoidable and then this was possible even when working with strong acids. Consequently, the separations of

pheophytin into phytylpheophorbides *a* and *b* with 30 per cent hydrochloric acid

the methyl pheophorbides *a* and *b* with 17 per cent hydrochloric acid and

the free pheophorbides *a* and *b* with 16 per cent hydrochloric acid

depend upon the same method and are to be considered among its most important applications. There is here a question (as, in what follows, with phytochlorin *e* and phytorhodin *g*), above all, of the separation of products representing analogous phases of the systematic decomposition of the two chlorophyll components.

On saponification of the pheophorbides; namely, of various pheophytin preparations, with alcoholic potash, two series of compounds are obtained, which are both weakly acid and weakly basic: the phytochlorins, which are olive green to green in indifferent solvents and blue green to blue in acid solution and the phytorhodins which are blue to green in acid solution and beautifully red in neutral solution.

Originally, extracts that contained chlorophyll formed the initial material that was used in the investigation and in these there had occurred various alterations, especially allomerization of the chlorophyll. These alterations may be accurately explained to-day. Consequently, there was not formed simply a chlorin and a rhodin from each of the two components but a whole host of compounds of the two groups. Ester acids of the two groups were also obtained; namely, phytochlorins by the direct action of alkalies upon the chlorophyll-containing extracts and phytorhodins by the treatment of the chlorophyllins with alcoholic hydrochloric acid.

Seven representatives of the phytochlorin series, designated chlorins *a* to *g*, and ten members of the phytorhodin series, designated rhodins *a* to *k*, have been described. Of all these compounds only one of each, namely, phytochlorin *e* and phytorhodin *g*, has special importance. These are the cleavage products that are formed by the smoothest decompositions and are, therefore, called the normal cleavage products of the respective chlorophyll components. Most of the other chlorins and rhodins are of little interest as chlorophyll derivatives. They are adduced only as those materials by means of which the method of separation and determination was worked out and described.

The phytochlorins and phytorhodins are insoluble in water; they are more or less soluble in organic solvents. As weak acids they dissolve in alkalies, also in ammonia and bicarbonate, and are quantitatively extracted by these from an ethereal solution. They contain only acid groups that are capable of esterification; their esters are insoluble in alkali. All are weak bases, whose salts are completely decomposed by water.

Moreover, their basic properties are much more closely differentiated than their acid properties and they disclose a series of differences and gradations such as has heretofore not been observed in weak organic bases.

For their extraction from an ethereal solution by hydrochloric acid these compounds require acids of definite concentration and this must be used in excess. Practically, sulphuric acid and even phosphoric acid

Phytochlorin	Traces are extracted by HCl of the strength	Extracted very abundantly by HCl of the strength	Extracted almost completely by HCl of the strength
a	3.5 %	6.5%	7.5%
b	1.5	3.5	5.0
c	0.5	1.5	2.0
d	0.15	0.5	1.0

are less applicable because the concentrations required are quite high. Each individual substance has characteristic limits so that hydrochloric acid up to a certain strength extracts none, or only traces, of it from its ethereal solution, a somewhat more concentrated solution of acid extracts a large part of it, and finally, a still greater concentration practically extracts it all with a single thorough shaking. It is self-evident that the concentration of the ethereal solution and the amount of hydrochloric acid exert considerable influence upon the result, and these factors must be considered in practical application. Of course, the limiting values approach each other the more closely the stronger the bases happen to be if one does not take into account the relative concentration of the hydrochloric acid but its percentage content. The following table indicates the limits for the first phytochlorins:

The following, normal, working conditions are fundamental to the observations: 1–2 cc. of an ethereal solution which contains 0.1 g. of

substance in 100 cc. is thoroughly shaken once with an equal volume of hydrochloric acid. The distribution of phytochlorin *a* and *b* between ether and hydrochloric acid was quantitatively investigated by using such solutions.

Three portions, each of 0.100 g., were dissolved in 100 cc. of ether and each was thoroughly shaken for five minutes with 100 cc. of hydrochloric acid. The layers were then separated and the ethereal solution was washed with a little water and carefully evaporated; the residue was dried to constant weight in a vacuum over sulphuric acid. The following table specifies the portion that was taken up by the different hydrochloric acids:

Phytochlorin	Strength of hydrochloric acid in per cent	Per cent of substance extracted
a	8.0	84.1
a	7.0	73.8
a	6.0	60.7
b	5.5	74.4
b	4.0	54.7
b	2.5	26.4

The possibility of separation depends upon these different basicities. Phytochlorins *a* and *b* again serve as examples for the description of the manner of procedure. They are obtained mixed with more weakly basic substances. If the ethereal solution[2] is shaken repeatedly with 4 per cent and 7 per cent hydrochloric acid and, if the two acid solutions are washed thoroughly with ether in order to again extract the weaker bases that have been taken up in every case, a considerable portion of *a* and *b* can be isolated in a rather pure state.

A second method of carrying out the fractionation is much better than this. Its principle consists in extracting the compounds in the order of their basicity with such weak acids that only minimum traces of the next weaker bases can be dissolved. Naturally, the strongest acids that will satisfy this condition must be used. Thus, in the given case, phytochlorin *b* is isolated by means of 3 per cent hydrochloric acid. But since *b* is not quantitatively extracted from the ether by this, it becomes necessary to separate an intermediate fraction by re-

[2] It is, of course, not necessary to work with the solution of the mixture but the goal is attained more difficultly when the powdered form is used.

peated extractions with 4.5 per cent acid before the extraction of *a* is begun. For this, 6 per cent acid is used. In this method, also, the hydrochloric acid solutions (3 or 6 per cent) of phytochlorin are to be washed with ether. The substances are then obtained in a pure state merely by a single fractionation. The method is altered somewhat in order to purify perfectly the chlorophyll derivatives when they are already at hand in a rather pure state as, for example, that in which they would be obtained by the first method which, as regards yield, is a more advantageous method of separation. Thus, phytochlorin *a* is dissolved in ether, the solution is washed repeatedly with 4.5 per cent hydrochloric acid, and then the base is extracted with 6.5 per cent acid.

The already purified substances are then resolved again in the same way by fractionation and their identity proved.

The method is particularly applicable to these colored pigments. Here it is only an easy matter to find out in each case the acids that are suitable for the isolation and to estimate the extracted portion by means of the intensity of the color of the extract. It is important, in this connection, to compare the different extracts of a given substance with regard to their hues in order to determine whether it is pure.

A similar separation of the chlorophyll derivatives by means of alkali is not possible since, in general, their alkali salts are split hydrolytically to a far less extent, but they may be fractionated, though not nearly so well, by means of alkalies according to the customary methods. Thus—for example, in the purification of phytorhodin *f* which, after a rough fractionation with hydrochloric acid, was still mixed with very similar basic compounds—the procedure was such that the amount of alkali necessary for neutralization was diluted and then divided into a series of portions and the ethereal solution successively extracted by shaking with these portions. The fractions, which were again brought into ether, were then estimated by means of their color.

Such a supplement to the fractionation that had been carried out in acid solution was but seldom necessary. The compounds were almost always obtained in a pure condition so that they crystallized well from an ethereal solution without further purification. The colorless impurities of the chlorophyll derivatives, such as fats and waxes, which are separated with difficulty by mere recrystallization, are removed by solution in dilute acid.

The fractionation method is just as valuable for *qualitative analysis;* namely, for the determination of the derivatives of chloro-

phyll, as it is for their separation. The reaction products of all possible reactions that give rise to more or less basic materials may even be investigated in a test tube dropping funnel by shaking the ethereal solutions thoroughly with hydrochloric acid of graduated concentrations. It is observed whether the products are mixtures or pure, and they may be arranged according to their basicity. The slightest changes of the chlorophyll derivatives described, when stored or dried with heat, were in this way made conspicuous.

In order to employ the method here described for the investigation of the phyllins, they are decomposed by acid and the resulting porphyrins are tested; from the basicity of the porphyrin and from its purity conclusions may be drawn regarding the original phyllin.

2. The Hydrochloric Acid Number.

The phytochlorins *a, b, c,* and *d,* which were mentioned in the examples for the hydrochloric acid method, are of no significance in the decomposition of chlorophyll. The more important, magnesium-free, chlorophyll derivatives are adduced in the three following tables which give us information as to their basic properties.

That concentration of the hydrochloric acid, which under the usual working conditions extracts a considerable amount of the substance from ether, is so significant for preparative purposes that it should be specially characterized. Consequently, in the tables and in what follows, the name "hydrochloric acid number" is given to the percentage content of those acids that extract approximately two-thirds of the dissolved substance from an equal volume of ethereal solution on thorough shaking.

For this test 0.02 g. in 100 cc. of ether may be advantageously employed; or, if the solubility is less than this, use a saturated, ethereal solution.

The hydrochloric acid number may be sufficiently accurately measured in a test tube. It expresses a phenomenon that is characteristic of the chlorophyll derivatives but which has not yet been observed in connection with other compounds; namely, that the ratio in which the base is distributed between equal volumes of ether and aqueous acid may be changed to an extraordinarily large extent by changes in acid concentration and, consequently, that a variation of the acid content by a few per cent suffices to shift the value of the ratio from approximately nothing to almost infinity. Consequently, it is unimportant for

a description of the basic properties that the exact distribution between the ether and hydrochloric acid is in most cases not stated here, but that only the point is given where there is an abundant transfer from ether to the acid.

Further application of Willstätter and Mieg's method of determination to the compounds that are formed as a result of the removal of

1. *The Pheophorbides.*

	Traces extracted by hydrochloric acid of the strength	Hydrochloric acid number	Almost completely extracted by HCl of the strength
Pheophytin *a*	25 per cent	29	32 per cent
Pheophytin *b*	30 " "	35	— " "
Methyl pheophorbide *a*	13 " "	16	18 " "
Methyl pheophorbide *b*	17 " "	21	23 " "
Pheophorbide *a*	12 " "	15	17 " "
Pheophorbide b	16 " "	19.5	22 " "

2. *The Phytochlorins and Phytorhodins.*

	Traces are extracted by HCl of strength	Hydrochloric acid number	Almost completely extracted by HCl of strength
" *f*	7 " "	10	12 " "
" *g*	8 " "	10–11	12–13 " "
Phytorhodin *g*	6 " "	9	11 " "
" *i*	11 " "	15–16	20 " "
" *k*	9 " "	14–14½	18 " "
Phytochlorin *e*	½ per cent	3	4– 5 per cent

iron from hemin proved successful. According to Willstätter and M. Fischer[3] the hematoporphyrin group is a series of compounds that are very closely related to each other and their differentiation and separate identification is important. This can be done with the aid of the hydrochloric acid number, even for the smallest quantities, whereas no other means of doing this is known.

[3] Unpublished.

3. *The Porphyrins.*

	Traces are extracted by HCl of strength	Hydrochloric acid number	Almost completely extracted by HCl of strength
Glaucoporphyrin	2 per cent	4–5	6 per cent
Cyanoporphyrin	1 " "	4	5 " "
Rhodoporphyrin	2 " "	3	4 " "
Erythroporphyrin	– " "	—	– " "
Rubiporphyrin	2½ " "	4½	6½ " "
Pyrroporphyrin	½ " "	1½	3 " "
Phylloporphyrin	1/10 " "	¾	1½ " "
Etioporphyrin	1 " "	3	4 " "

4. *The Porphyrins from Hemin.*

	Traces are extracted by HCl of strength	Hydrochloric acid number	Almost completely extracted by HCl of strength
Hematoporphyrin	0.033 per cent	0.1 – 0.15	0.4 per cent
Heminoporphyrin	0.033 " "	0.15	0.4 " "
Hemidoporphyrin	0.2 " "	1.0	2.5 " "
Hemoporphyrin	0.15 " "	0.75	2.0 " "
Mesoporphyrin	0.5 " "	1 – 1.5	3.0 " "
Etioporphyrin	1.0 " "	3.0	4.0 " "

3. The Distribution Number.[4]

The hydrochloric acid number does not fully characterize the basicity although it is sufficient for the identification of many chlorophyll derivatives; a more exact number is required to distinguish between compounds that are approximately the same in their basicity.

The behavior of such a basic compound would be more completely described by means of its distribution expressed as a function of the relative volumes of ether to acid for various concentrations of the acid. Especially at the concentration of the hydrochloric acid number should the dependence of the distribution of the base between acid and

[4] Unpublished.

ether upon the relative volumes of the two layers be determined. The hydrochloric acid number could be derived from this distribution.

In order to obtain this more exact expression of the basic character by means of a constant it was sufficient to ascertain the distribution of the base for only a single relative volume of ether to hydrochloric acid, a single concentration of the hydrochloric acid and a single concentration of the ethereal solution.

For practical reasons about the same strength of acid is chosen as in the case of the hydrochloric acid number. Not the relative volumes 1 : 1, however, but the volumetric ratio, 1 of hydrochloric acid to 10 of ether, and a very low concentration of the substance in ether are used.

That fraction of a substance, expressed in per cent, which is extracted under certain conditions from an ethereal solution by hydrochloric acid of a definite concentration is designated as its distribution number.

These conditions are: 3 mg. of substance in 1 l. of ether and 100 cc. of the hydrochloric acid layer.

The concentration of hydrochloric acid can, of course, be not only that expressed by the hydrochloric acid number but, on the contrary, there is more often chosen, for comparisons, any other strength of acid suitable for the extraction. For the comparison of compounds of approximately the same basicity the same strength of hydrochloric acid is employed; for example, 0.5 per cent for phylloporphyrin and pyrroporphyrin, the hydrochloric acid numbers of which are 0.75 and 1.5.

Although the hydrochloric acid number gives a suitable concentration of acid for the extraction of a compound, the distribution number plainly shows the difference between the basic properties of compounds that are similar to each other and makes possible certain identification of a chlorophyll or a hemin derivative.

Determination: Three mg. of the substance, which has been dried to constant weight, are dissolved in a few cubic centimeters of ether-containing, concentrated hydrochloric acid and transferred to 1 l. of ether by neutralization with ammonia, the solvent power of the ether having been increased by the addition of 50 cc. of alcohol. The alcohol is removed by washing with 5 l. of distilled water and the volume of the solution is made up to 1 l. by the addition of ether. The ethereal solution is strongly shaken for a minute with 100 cc. of suitable hydrochloric acid, the per cent content of which has been determined by

titration or with the hydrometer. The increase in volume (about 10 cc.) of the hydrochloric acid layer due to the ether taken up is measured and kept in mind.

Either (a) the hydrochloric acid solution of the portion that remains in the ether, or better, (b) a new comparison solution of the same substance, may be used for the colorimetric determination of the extracted substance.

In the former case the residue that remains in the ether is extracted with a more concentrated hydrochloric acid and diluted with water, containing ether, to the same concentration of acid as that of the first extract.

Distribution Numbers of Some Chlorophyll and Hemin Derivatives.

Substance	Preparation	Concentration of HCl in per cent	Distribution number
Phylloporphyrin	from phytochlorin *e*	0.5	33.4
	from phytorhodin *g*	0.5	37.1
	by-product of rhodophyllin (preparation of Willstätter and Fritzsche, 1909)	0.5	35.7
	from methyl chlorophyllide *b*	0.5	34.6
Pyrroporphyrin	from chlorophyll *a* by way of pyrrophyllin	0.5	4.8
	from phytorhodin *k*	0.5	3.8
	from phytorhodin *i*	0.5	4.4
Glaucoporphyrin	from glaucophyllin	3.5	15.4
Rhodoporphyrin	from rhodophyllin	3.5	18.1
Rubiporphyrin	from rubiphyllin	3.5	7.4
Phytorhodin *k*	from allomerized chlorophyll *b*	15.0	7.8
Phytorhodin *i*	from allomerized chlorophyll *b*	15.0	4.2
Mesoporphyrin	from mesohemin	0.5	12.9
Mesoporphyrin	obtained by the method of Nencki and Zaleski	0.5	11.8
Phonoporphyrin	from hematoporphyrin	0.5	23.5
Etioporphyrin	from chlorophyll	3.0	40.2
Etioporphyrin	from hemin	3.0	43.1

In the second case, another 3 mg. sample of the substance is dissolved in so much ether-containing, concentrated hydrochloric acid (20–35 per cent, according to the solubility) that, when diluted with water, which has been saturated with ether, to the volume of the extract that is to be determined there results a hydrochloric acid solution having the same per cent content as this extract.

If the depth of the layer of the solution under examination is h_1 and that of the comparison solution (according to (b) h_2, then the distribution number is $\frac{h_2}{h_1} \times 100$.

Example: The distribution number served as a test of the purity of a new porphyrin, hemoporphyrin that had been obtained by the decomposition of hemin. It was split up into four fractions by extraction from ether with dilute hydrochloric acids. These fractions agreed exactly as to their distribution numbers and were therefore identical.

Another application of the distribution number consists in the comparison of preparations of a substance made in different ways. The above table makes this evident and at the same time gives clues to differences found in the determination.

XV. THE PHEOPHORBIDES A AND B.

1. Separation of Pheophytin into its Components.

The isolated chlorophyll components, by cleavage with acid, yield their pure magnesium-free derivatives. It is not necessary for their formation first to isolate the chlorophyll preparations; chlorophyll solutions that have been fractionated according to the procedure of Willstätter and Isler may be employed just as well.[1] For example, a petroleum ether solution of chlorophyll *a*, such as remains on transference of the chlorophyll to 95 per cent methyl alcohol, is freed entirely from any admixture of *b* by washing it with 90 and 95 per cent methyl alcohol, and is then decomposed with alcoholic oxalic acid. The crude pheophytin *a*, in a filterable form, is precipitated after concentration and repeated evaporation with alcohol.

The preparation of phytyl pheophorbides from the chlorophyll components is tedious and expensive; the fractionation of the pheophytin according to the method of Willstätter and Mieg is much more important.

Willstätter and Isler[2] employed this method for the isolation of pheophytin *b*, and although they could not isolate the *a* component (which is easily saponified by extraction with the requisite strength of hydrochloric acid) as such, they prepared free pheophorbide *a* from it. The method has been so improved that it permits the two phytol compounds to be obtained in an unsaponified state even in large quantities.

The new method.[3]

The fractionation of pheophytin requires at least 27 per cent hydrochloric acid. This strong acid dissolves considerable ether and, in doing so, becomes so warm that the hydrolysis of the portion that goes into the acid cannot be avoided. Consequently, hydrochloric acid that has been previously saturated with ether and cooled is used. This, in fact, is often made stronger than 27 per cent in order to complete the

[1] Ann. d. Chem. 390: 321, 323. 1912.

[2] Ann. d. Chem. 390: 324. 1912.

[3] Unpublished.

fractionation more quickly. As a consequence of the preliminary treatment of the acid with ether a more concentrated pheophytin solution can be subjected to the separation.

The extraction of the *a* component takes place with 30 per cent (the purification of *b*, with 31 per cent) hydrochloric acid, which is prepared in an ether-saturated condition in the following manner.

Seven liters of concentrated hydrochloric acid (specific gravity 1.19; that is, 37.5 per cent at 15°) are cooled from 15° to 0–1° by the introduction of pieces of ice and stirring. When this temperature is just reached the hydrometer indicates 31.5 per cent of hydrochloric acid or, on warming a sample to 15°, exactly 30 per cent. The cold acid is shaken in three portions, each with an equal volume of ether. It takes up 90 per cent (by volume) of ether and, in doing so, its temperature rises to 28–29°.

In the case of the 31 per cent acid, the 37 per cent acid is cooled from 15° to only 2–3° by the addition of ice. The hydrometer then indicates 32.3 per cent; that is, as much as 31 per cent on warming a sample to 15°.

This second acid, upon saturation with ether, takes up 93 per cent (by volume). The hydrochloric acid-ether mixtures are cooled again to 0° for use.

Twelve grams of comminuted pheophytin, having a component ratio of 2.5, are shaken by machine with 2 l. of ether until dissolved. First, a purification by washing the solution with 100 cc. of 22.5 per cent hydrochloric acid while the ethereal solution is still in the shaking flask is recommended; this procedure often precipitates a trace of the impurity that causes emulsions and separates, at the same time, some admixed alcoholyzed pheophytin. All the liquor is filtered into a separatory funnel, the hydrochloric acid layer is separated and the volume of ether is made up to 3 l.

The separation is then accomplished by a series of extractions with the 30 per cent acid that has been described. The separate, deep blue extracts, obtained with each two liters of acid by careful shaking, are each, even if turbid because of the ether, placed as quickly as possible in a second separatory funnel which contains 0.5 l. of ether, and freed from a slight admixture of the *b* component by shaking for a short time. The acid layer is then allowed to flow immediately into a third separatory funnel which contains considerable water whereupon all the *a* component passes into the ether which separates in abundance and suffices for its solution.

After each extraction with 30 per cent hydrochloric acid, the volume of the ethereal solution is replenished with 500 cc. of the wash ether from the previous extraction, which contains a little *b*; the acid that was used still took up 25 per cent (by volume) of ether since it had been saturated with ether only at about 30°.

In the same manner eight extractions were made, washing quickly each time (the wash ether continually becoming poorer in *a* and more brown) and diluting at once. The ethereal solution of the *a* component, in all 5–6 l., was collected. It contains but very little, free pheophorbide which can be easily removed by washing with 0.5 l. of 25 per cent hydrochloric acid.

The residual, ethereal solution of the *b* component (about 3 l.) is still impure. It must be extracted 4–5 times, using 1 l. of the previously prepared 31 per cent acid each time. The first wash liquors are still bluish green, the last are almost pure green and yield, when transferred to ether, a reddish brown solution the phase test of which is brownish red to red. The volume of the *b* solution is not increased; it amounts at the end of the procedure to about 2 l. Its color is a deep reddish brown, the phase with methyl alcoholic potash is a pure red; free pheophorbide *b* is not formed by the cold acid.

The ethereal solutions of the two components are finally washed with water until the acid reaction disappears and, after drying for a short time with sodium sulphate, each is evaporated to about 0.5 l. They are then filtered again into a 0.75 l. flask, finally evaporated with rotation of the flask to about 100–150 cc., and the products precipitated with alcohol; namely, *a* with 0.5 l. of 85 per cent, *b* with 0.5 l. of 90 per cent, alcohol. The precipitates separate in an easily filterable state and the mother liquors are very clear. The *b* component is obtained in an absolutely pure form; the *a* component, on the other hand, has an admixture of *b* which is easily detectable by means of the cleavage test. This, in any case, is less than 5 per cent.

Yield: *a*, 6.8 g.; *b*, 3.2–3.3 g.; the losses in all the purifying operations, therefore, amount, on the whole, to only about 15 per cent.

In order to obtain pheophytin *a* also in an entirely pure state, the fractionation with hydrochloric acid is repeated in the following manner.

Ten grams of the above described preparation are redissolved in 2.5 l. of ether. As in the case of the first separation eight extractions are made, each with 2 l. of cold 30 per cent hydrochloric acid that has

been saturated with ether, the volume of the ether being kept constant until the sixth extraction, although it may decrease towards the end of the procedure. About 10 per cent of the material, a mixture of *b* with *a*, remains in the ether with a brown color.

Each separate acid extract of pheophytin is placed at once in a large separatory funnel through which a constant stream of water is carefully allowed to flow. The diluted acid runs off with a greenish color and takes some hydrolyzed pheophytin along with it. It is always advantageous to free the ethereal solution from this by washing once or twice more with 25 per cent hydrochloric acid (with 0.5 l. each time) and then with water till the reaction is neutral. The ether is then finally evaporated down to 1 l., filtered and further concentrated to 150 cc. The pure phytyl pheophorbide *a* is then precipitated with 500 cc. of 85 per cent alcohol, shaking vigorously so as to avoid caking of the voluminous precipitate.

The yield amounts to 8.6 g.

2. Fractionation of the Methyl Pheophorbides.[4]

The initial material is a mixture of the methyl chlorophyllides. After this has been resolved into its two components as a consequence of their different distributions between ether-petroleum ether and methyl alcohol, the pure methyl pheophorbides can be very easily prepared from them, and with quantitative yields, by the action of acid. The ethereal solution of methyl pheophorbide *a* is shaken a few minutes with 10 per cent (that of *b*, with 15 per cent) hydrochloric acid; a little longer than is necessary for a complete change of color so that the preparations become wholly ash-free. Upon moderate concentration of the ethereal solutions, the difficultly soluble pheophorbides form beautiful crystals.

But the separation of the magnesium-free and, therefore, acid-stable derivatives is, in consequence of their somewhat different basicity, much more easily performed and it is more exact. This is done by the application of the method of Willstätter and Mieg. It is, consequently, more rational first to remove the magnesium from the mixture of the two methyl chlorophyllides by means of acid and then to undertake the fractionation of the methyl pheophorbide mixture that is formed by means of 17 per cent hydrochloric acid. This concentration corresponds to the hydrochloric acid number of the *a* component, while *b*

[4] Ann. d. Chem. 387: 370. 1912.

is only slightly extracted by 16 to 17 per cent acid and not abundantly so until the strength of the acid reaches 21 per cent.

Two grams of a methyl chlorophyllide mixture (containing about 2 parts *a* and 1 part *b*) is dissolved in a little pyridine and transferred to 4 l. of ether. It is necessary to work with such a dilute solution on account of the slight solubility of the methyl pheophorbide *b* that is formed. A change of color and decomposition of the complex compound results when the ethereal solution is shaken vigorously with 17 per cent acid while extraction of the methyl pheophorbide *a* begins at the same time. The acid is colored a deep blue; six extractions, each with 1 l. of acid, are required, till the extract shows only a pale blue color. The hydrochloric acid has a gradual hydrolytic action upon the easily saponifiable ester group so that the hydrochloric acid solution is not allowed to stand unnecessarily but is allowed to flow from the separatory funnel into a second one where it is washed with ether in order to separate a trace of methyl pheophorbide *b* that is carried along; a little *a* is lost in this washing. This wash ether from each hydrochloric acid extract is poured back into the first separatory funnel, thus keeping the volume of the ethereal layer constant. Each hydrochloric acid extract is allowed, immediately after it has been washed, to flow into a third separatory funnel where it is washed with an equal volume of water and with 0.01 *N* KOH which, by working rapidly, removes but very little of the free pheophorbide. The olive green, ethereal solution is then dried and slowly evaporated to about 100 cc.; the product is so pure that it need not be isolated in fractions. During the concentration the methyl pheophorbide *a* crystallizes in single, perfect crystals which have a beautiful, violet black luster.

In order to purify the *b* component, the ethereal solution, after the extraction of the main *a* portion, is washed twice more with some 18 per cent hydrochloric acid, then with water, and evaporated. The pure methyl pheophorbide *b* crystallizes in metallic lustered, dark gray plates as soon as the solution becomes warm at the start of the concentration.

The yield depends only upon the composition of the methyl chlorophyllide mixture that has been used; in the case of the ratio stated above it amounts to 1 g. of methyl pheophorbide *a* and 0.5 g. of *b*.

The ethyl pheophorbides may be separated by means of the hydrochloric acid method in the same manner as the methyl compounds; 17 per cent acid is suitable in this case also.

3. Substitution of the Alcohol Radicle in Pheophytin by Means of Hydrochloric Acid and Alcohol.[5]

The use of chlorophyllase offers, in the case of chlorophyll, the only possibility of bringing about replacements of the phytol ester group while limiting these exchanges to this group alone. Acid media are excluded on account of the sensitiveness of the magnesium complex; alkalies, on account of the instability of the lactam group and also on account of the saponifiability of the second ester group. On the other hand, pheophytin, which is only sluggishly transformed through the action of chlorophyllase, may have its phytol ester group alone replaced with the aid of acid; that is, the phytol may be replaced by simple alcohols in the presence of a little hydrogen chloride according to the method of A. Haller,[6] or saponification with strong aqueous hydrochloric acid can take place. Important methods of preparation, of which the method for the preparation of methyl pheophorbide is first described, depend upon this.

Pheophytin dissolves in methyl alcohol only with difficulty, even when heat is applied; in the presence of only a little hydrochloric acid, however, it dissolves much more easily. The liquid has a strong dark red fluorescence and a deep blue color, which is remarkably consistent for the pheophorbides of the *a* and *b* series, which are distinguished only by a more bluish tinge of the *a* and a more greenish tinge of the *b* and by a darker tint of the fluorescence of the *a*.

10 g. of pheophytin are crushed as finely as possible, shaken with 1 l. of methyl alcohol and 100 cc. of 22 per cent methyl alcoholic hydrochloric acid are added, while stirring, so that the material does not cake. Methanolysis is completed in 1 hour when boiling under a reflux condenser. A sample is then, after transferring to ether, quantitatively extracted by 22 per cent hydrochloric acid as long as this is not colored at all by the ethereal pheophytin solution (basicity test).

The liquor is cooled somewhat, poured into 4 l. of ether, and the methyl alcohol is washed out with water. A portion of the methyl pheophorbide mixture, in which the *b* component is much more concentrated, now crystallizes from the solution even before it is concentrated. The filtrate is evaporated to about 300 cc., whereupon a beautiful crystallization of the methyl compound, which consists mainly of

[5] Unpublished.

[6] Compt. rend. 143: 657. 1906; 144: 462. 1907; 146: 259. 1908. A. Haller and Youssoufian, Compt. rend. 143: 803. 1906.

the *a* component, separates. Finally, upon stronger concentration, a smaller third fraction is obtained, which consists almost wholly of methyl pheophorbide *a*. These separate crystallizations are specially suited for fractionation according to the above described hydrochloric acid method on account of the enrichment in their components.

The total yield amounts to 6.2 g.; that is, 90 per cent of the theoretical.

The ethereal mother liquor, which could be decolorized by 22 per cent hydrochloric acid, contains the phytol which has been split off, although not in an unchanged form. The 3.1 g. of oil that remain on evaporation is, as distinguished from phytol, only moderately soluble in methyl alcohol and is shown, by the methoxyl determination, to consist chiefly of phytylmethylether.

4. Formation and the Separation of the Free Pheophorbides.[7]

Free pheophorbide, a mixture of the two components, is formed upon acidification of chlorophyllide or by hydrolysis of methyl chlorophyllide with hydrochloric acid. In this case, the 16 per cent acid, suitable for fractionation of the mixture, is not sufficiently strong for the hydrolysis; neither does it require such concentrated hydrochloric acid as is necessary for the hydrolysis of the phytyl compounds.

Two grams of a mixture of the two methyl chlorophyllides are dissolved, with the aid of pyridine, in 4 l. of ether and the ethereal solution is extracted 3 times, with 0.5 l. of 25 per cent hydrochloric acid each time. The acid solution is allowed to stand for 2 hours at room temperature. The hydrolysis, the course of which is followed by extracting samples with ether and adding 0.01 *N* KOH, is then practically ended. The substance is extracted with ether and precipitated, by the introduction of dry ammonia gas, as a brown, flocculent, ammonium salt, which is collected with talc and filtered; some residual ester is found in the filtrate. The carboxylic acid is again liberated by extracting the talc on the suction filter at once with acetone which contains a little hydrochloric acid. The filtrate is introduced into ether, the acetone is washed out and fractionation of the mixture is carried out according to the following procedure. The yield amounted to 1.0 g. of pheophorbide *a* and 0.55 g. of *b*.

Preparation of Pheophytin by Hydrolysis. The hydrolysis of pheophytin, by means of which the two free pheophorbides are changed to

[7] Ann. d. Chem. 387: 378. 1912.

most easily accessible and very fine initial materials for further inve tigations, is practically more important.

The pheophytin must consist of the two pure components only; *i.e* it must produce no weakly basic compounds in the cleavage test, els allomerized derivatives of the two components would remain admixe with them.

The ethereal solution of pheophytin is introduced, without coo ing, into concentrated (34–35 per cent) hydrochloric acid; fc example, 4 g. of pheophytin with about 800 cc. of ether into 2 l. c acid. A test shows, even after ¾ to 1 hour, that the carboxylic aci is formed quantitatively. The hydrochloric acid is now mixed wit 800 cc. of water and the phytol removed by extraction with ethe The acid solution is then diluted further and the pigment extracte with about 7 l. of ether; too little ether must not be used, else th pheophorbide would precipitate. After concentration of the etherea solution to 5 l., the pheophorbide *a* is extracted from the mixture o carboxylic acids 5 times, using 1 l. of 16 per cent hydrochloric aci each time. The separate hydrochloric acid extracts are washed wit 200 cc. of ether and the wash ether is returned each time to th ethereal mother liquor. This, in order to remove the last traces of *a* was shaken out twice more with 250 cc. of 17 per cent hydrochlori acid. For preparative purposes it does not pay to work up this ex tract. After the purification with 17 per cent acid, pure pheophorbid *b* remains; its solution, after washing with water and drying, is evapo rated (yield, 0.9 g.). The *a* component requires only to be trans ferred from the hydrochloric acid solution to ether by dilution wit water and crystallizes completely on concentration (1.6 g.).

5. Description of the Pheophorbides.

The Phytyl Pheophorbides.[8]

The *a* component, $(C_{32}H_{32}ON_4)$ $(COOCH_3)$ $(COOC_{20}H_{39})$ + $\frac{1}{2}H_2O$ dissolves with difficulty in absolute alcohol when cold; when warm rather easily, with a sepia brown color; a small water content strongly depresses its solubility. The substance separates in coarse floccule from its alcoholic solution upon rapid cooling; when slowly cooled, i separates in a more granular form and in forms which appear unde the microscope as arborescent-branched crystalline aggregates withou sharp borders. Ether easily dissolves pheophytin *a*, although slowly

[8] Ann. d. Chem. 390: 332. 1912.

benzol and acetone dissolve it very easily; chloroform and pyridine, exceedingly easily, and petroleum ether, very difficultly. Concentrated solutions are olive brown, the more dilute ones are olive green, similar to those of phytochlorin *e*, but they are distinguished from these by having a feeble red fluorescence.

It dissolves with considerable difficulty in glacial acetic acid when cold but, when warm, glacial acetic acid dissolves it moderately with a violet tinged brownish color. On the other hand, it dissolves very easily in formic acid, even when cold, with an indigo blue color.

When dried, the phytyl compound forms bluish black, tough, tenaceous, somewhat waxy lumps. It frits in a melting-point tube at 110–114°, and it then becomes viscous at 120°.

Pheophytin *a* gives the yellow phase with concentrated methyl alcoholic potash but this disappears much more quickly than in the case of the magnesium compound. A chlorophyll green color appears also in the alkaline solution of pheophytin, which is explained by the formation of a complex in which potassium has the same function as magnesium in chlorophyll.[9] This formation of a complex takes place only in highly concentrated alkali.

The hydrochloric acid number of the *a* component is 29. The acid solution is pure blue in color. Upon placing a layer of concentrated nitric acid beneath a cooled ethereal solution, the ether assumes a beautiful, violet blue color; the *b* component gives, in this reaction, a green color for only a very short time, which changes to a red brown as the result of a radical change.

Pheophytin *b*, $(C_{32}H_{30}O_2N_4)(COOCH_3)(COOC_{20}H_{39})$, is a much weaker base; its hydrochloric acid number is 35 and its acid solutions are green.

The phase produced with alcoholic lye is beautiful red, but of short duration.

The *b* phytyl compound forms a greenish black mass, which is more brittle than the *a* compound and can be more easily ground to a grayish black powder; on heating, the substance frits at 148–152°, becomes viscous and intumesces at 160–170°.

Pheophytin *b* is difficultly soluble in cold absolute alcohol; in warm alcohol it is rather difficultly soluble; in fact, it is much more difficultly soluble than *a*, dissolving with a reddish brown color having a greenish yellow tinge and a feeble brownish red fluorescence. In

[9] See Chapter XVI, section 3.

ether, in which it dissolves considerably, it shows a very similar color, which reminds one of that of phytorhodin. The substance is soluble only with very great difficulty in 95 per cent alcohol, even when this is hot; in cold petroleum ether it dissolves with extreme difficulty and only very slightly on heating the same.

Pheophytin *b* is consequently more easily precipitated than is *a* from its alcoholic or acetone solutions by means of petroleum ether.

It precipitates as fine, beautiful grains, less flocculent than *a*, from its warm alcoholic solution; likewise, only dendritic aggregates or rounded grains were to be observed under the microscope.

The Methyl Pheophorbides.[10]

The *a* component, $(C_{32}H_{32}ON_4)$ $(COOCH_3)_2$, crystallizes in sharply defined rhombic plates, which are often recognizable with the naked eye, and in prisms which show swallow-tail twin formations (Plate IV, Fig. 1). They have a beautiful, violet black luster and, when ground, form a dark violet powder. Under the microscope, the thinnest crystals appear brownish gray while the thicker ones are brownish yellow to brownish red.

The methyl ester dissolves in ether with great difficulty, imparting an olive brown color, which is olive green in thin layers, showing a dark red fluorescence. The substance is insoluble in petroleum ether; it is almost insoluble in cold alcohols, though, on heating, it is quite soluble; in chloroform it is very easily soluble, more easily so than in pyridine.

The hydrochloric acid number of methylpheophorbide *a* is 16; that of *b*, 21.

Methylpheophorbide *b*, $(C_{32}H_{30}O_2N_4)$ $(COOCH_3)_2$, is very similar in crystal form and solubility to the corresponding *a* derivative. The grayish black crystallizations from ether always consist of large, singly formed, and sharply outlined rhombohedra (Plate IV, Fig. 2), while the plates of methyl pheophorbide *a* frequently show rounded forms and group themselves in aggregates. In transmitted light under the microscope, the crystals of methyl pheophorbide *b*, with increasing thickness, are olive green, light brown and brown, while in the case of *a* the color deepens more to red. In solutions, the relation of the colors appears reversed; methyl pheophorbide *a* is olive brown in

[10] Ann. d. Chem. 387: 373. 1912.

ether; *b*, on the contrary, shows a reddish brown color with a greenish yellow tinge.

The *b* component is extremely difficultly soluble in ether and also is only very slightly soluble in boiling alcohols.

The Free Pheophorbides.[11]

The *a* component, ($C_{32}H_{32}ON_4$) ($COOCH_3$) (COOH), crystallizes excellently from ether and from alcohol in bluish black, lustrous, sharply defined, rhombic plates, the acute angles of which are often truncated. The powder, also, is bluish black and not dark violet as is that of the methyl ester. The thin crystals are olive green under the microscope while the thicker ones are olive brown to brown. The substance is difficultly soluble in cold, absolute alcohol, though it is easily soluble in warm, absolute alcohol; in ether it is rather difficultly soluble; in acetone easily; in pyridine and chloroform very easily; it is, on the contrary, insoluble in petroleum ether. In the usual solvents, the carboxylic acid has the same olive green to olive brown color as has its methyl ester, and also the distinct dark red fluorescence.

The pheophorbide is soluble with extreme ease in formic acid, forming a beautiful, blue colored solution that is violet red and red in transmitted light. The same color is brought out in an acetone solution by the addition of a small amount of hydrochloric acid; more acid is required for the corresponding color change in the case of the *b* component. Glacial acetic acid dissolves the substance, forming a violet tinged, brown solution, while the phytochlorins, because they are more strongly basic, dissolve in it with a blue salt color.

The ethereal solution (0.02 g. in 100 cc.) reacts quantitatively with 0.01 *N* ammonia and 0.01 *N* potash lye, which extract the pheophorbide with a brown color. Stronger potash lye (0.25 *N*) first precipitates flocculent potash salts, and then gradually dissolves them; very dilute solutions (0.001 *N*) are colored slightly olive green only. Even a 0.1 per cent soda solution and 1 per cent sodium bicarbonate, as well as 1 per cent disodium phosphate, precipitate the pheophorbide quantitatively as salt; 0.25 per cent disodium phosphate, on the contrary, extracts only a trace of the substance and gives no precipitate.

The lactam groups of the pheophorbides *a* and *b*, in distinction from those of the chlorophyllides, remain unaltered on being transferred to dilute alkalies and on long standing in alkaline solution, for

[11] Ann. d. Chem. 387: 381. 1912.

strong alkali still causes the brown phase and the reliberated pheophorbides still produce normal chlorin and rhodin.

The hydrochloric acid number of pheophorbide *a* is 15; that of *b* is 19–20.

The *b* component, $(C_{32}H_{30}O_2N_4)$ $(COOCH_3)$ $(COOH)$, is a stronger acid than *a*, to about the same extent that chlorophyllide *b* is stronger than chlorophyllide *a*. Pheophorbide *b*, for example, is extracted quantitatively from its ethereal solution (0.02 g. in 100 cc.) even by a 0.2 per cent sodium bicarbonate solution although this does not act at all upon the ethereal solution of the carboxylic acid *a*. The pheophorbides may be separated from each other on the basis of this difference. Pheophorbide *b*, as distinguished from *a*, is also quantitatively extracted by a 0.25 per cent solution of disodium phosphate and it even colors a 0.005 per cent soda solution rather strongly. The difference in color between the solutions in dilute and very dilute alkalies is very striking. The substance dissolves with a brownish red color in 0.01 *N* ammonia or in potassium hydroxide, with a pure brown color in a 0.001 *N* solution and with an olive green color in 0.0005 *N* alkali. These take up about one half of the pheophorbide from the ether in a single shaking. 0.00025 *N* ammonia extracts a fourth of the pheophorbide with a yellowish green color.

The pheophorbide gives a pure red phase with methyl alcoholic potash. This passes away much more quickly than in the case of the esters.

Pheophorbide *b* crystallizes from ether in very small, rhombic plates upon evaporation and in grayish black, lustrous rhombohedra, on cooling its alcoholic solution.

It is rather difficultly soluble in ether, but is much more easily soluble than the methyl compound; the solution is reddish brown and is tinged greenish yellow. It is rather easily soluble in warm ethyl alcohol; difficultly so in cold alcohol; easily, in acetone; very easily in chloroform and pyridine.

The Absorption Spectra.[12]

The three pheophorbides of each of the two series are very similar to one another in their absorption spectra. The spectra of the two pheophytin components are reproduced in the photographs of Plate IX and those of the methyl pheophorbides in the sketch of Plate VII.

[12] Paper XVII.

Measurements are given for several concentrations of the methyl pheophorbides *a* and *b*, which show sharper outlines of the bands and of the end absorption than do the phytyl compounds.

The a Component.

Between the Fraunhofer lines, B and G, the spectrum consists of 7, sharply separated bands. Absorption in the red, when compared with that of the chlorophyll component *a*, is almost unaltered but the absorption in the green is extraordinarily more intense; namely, adjacent bands, IV and V, of the chlorophyll (V and VI in the pheophytin) at the left and at the right of E have gained considerably in intensity.

The photograph scarcely permits it to be clearly recognized that the band in the red is accompanied by a narrow divided band in the red orange.

SOLUTION OF 0.030[13] G. IN 1 L. OF ETHER (0.001 MOL. IN 20 L.).

Layer, in mm.	2.5	10	40	80
Band I	672 . . . 661	678—654	685—646	687—641
" II	—	—	637 | 632	. . 631
" III	—	614 . 602	619— —599	621—597
" IV	—	—	565 . 552	566 . . . 552
" V	536 . 530	536 . . . 530	539—528	541—526
" VI	509 . 494	509 . . . 493	512—490	| 517—488
" VII	—	—	478 . 464	480 . . . 461
End absorption (VIII)	431 . 425—	433—	445 . 439—	. 452—

Sequence, according to intensity, VIII, I, VI, V, III, VII, IV, II.

The b Component. The spectrum is composed of eight, for the most part, sharp bands, one each in the red and in the orange, four in the green and two in the blue, in addition to which there is a strong end absorption (IX). The pheophytin spectrum, nevertheless, is less divided than that of chlorophyll, since it displays simple bands instead of the forked absorptions in the red and orange. Absorption in the green is intensified in the double band, IV and V, even if not entirely to the same extent as in the analogous derivatives of *a*.

The absorption spectra of the *a* and *b* components between the Fraunhofer lines, B and F, are of the same nature and are very similar to one another in spite of the striking difference in color of the two

[13] Trans. Calculation shows this to be 0.030 and not 0.30 as given in the German.

solutions. The band in the red and that in the orange is shifted towards the violet in the case of *b*; the outer band in the green is shifted considerably towards the red so that it is displaced close to the first band in the green (IV) and falls in the gap of the forked absorption of *a*. The narrow band, II, present in *a* is lacking in *b*. A bifurcated, very strong absorption in the indigo blue (bands VII and VIII) further distinguishes *b* from *a*.

Mixtures of the pheophytin components (for example, the preparations described by Willstätter and Hocheder[14]) derived from chlorophyll that has not been separated into its components consequently show all the absorption bands broadened and merged in comparison with the sharp delimitation and characteristic structure of the spectra of both components.

SOLUTION OF 0.030 G. IN 1 L. OF ETHER (0.001 MOL. IN 20 L.).

Depth of layer in mm.	2.5	10	40	80
Band I	659 . . . 652	664—646	670—640	674—636
" II	603 | 595	605 . . 593	608— —591	612—589
" III	—	563 . 552	565 . 551	566—550
" IV	537 | 531	·538 . . 530	539—529 . .	541—508
" V	523 | 517	524 . . 516	525—513	
" VI	—	—	494 . 483	494 . . 481
" VII	453 . . . 442			
" VIII	—427 .	457—	463—	465—
End absorption (IX)	415—			

Sequence of the intensities: IX, VIII, VII = I, IV, V, II, III, VI.

14 Ann. d. Chem. 354: 227. 1907.

XVI. THE PHYTOCHLORINS AND THE PHYTORHODINS.[1]

1. Preparation of Phytochlorin *e* and Phytorhodin *g* from the Pheophorbides.

Phytochlorin *e* and phytorhodin *g* are the most important products of the hydrolysis of pheophytin and the other pheophorbides.

Since these cleavage products are not formed simply by saponification of the two ester groups, but simultaneously by one of several possible transformations of the lactam groups, the reaction is, similarly to the saponification of chlorophyll, dependent to a large extent upon the conditions.[2] Different methods for the saponification of pheophytin are considered according to the main purpose which the hydrolysis serves; that is, whether this be the preparation of phytol or the isolation of the basic cleavage products. This saponification can be carried out in cold or hot alcoholic potash but never with dilute solutions; therefore, not with ethereal solutions of pheophytin. If these were used the more feebly basic, unstable chlorin *g* would be formed instead of the stable phytochlorin *e*, and the feebly basic phytorhodin *k* instead of phytorhodin *g*.

The first two of the three following methods are suitable for the preparation of phytol on a large scale.

The first method (*that of Willstätter and Hocheder*). Saponification by prolonged heating with alcoholic potash.

Pheophytin is boiled on a water bath with methyl alcoholic potash, using a reflux condenser and for each gram of pheophytin 5–7½ (most often 6) cc. of the potash solution.

This is prepared by dissolving 200 g. of ordinary stick alkali in 1 l. of methyl alcohol. Several hundred grams may be hydrolyzed at one time and the whole worked up for phytol.

The duration of the heating is dependent upon the state of subdivision of the pheophytin. With material that has been finely ground

[1] Papers III and XVI.

[2] See Chapter IV, section 2.

30 minutes is sufficient but, with lumps, 4–6 hours are required. Completion of the saponification may be recognized by the fact that no more lumps of unsaponified pheophytin are present, and secondly, that when a test sample is shaken with 20 per cent hydrochloric acid and ether none of its chlorophyll substance is taken up by these.

The yield and purity of the phytol are not disadvantageously affected by long heating, but the contrary is true for the basic compounds, since phytorhodin *g*, especially, will not endure heating with alcoholic potash and the longer it is heated the more it is changed into amorphous insoluble floccules.[3]

In order to render pheophytin, which cannot be pulverized, easily saponifiable Willstätter and Hocheder brought it into a state of fine subdivision by shaking it with considerable sea-sand and ether (4 l. for 33 g.) till solution was complete and then evaporating the fluid with the sand in a round flask to dryness; the residue was dissolved by boiling it in the same vessel.

Instead of doing this, it is also very advantageous to introduce the pheophytin in small portions gradually into the potash lye which has been heated to boiling. The clumping of the waxy product is thus prevented and saponification accelerated.

In order to extract the phytol, more than an equal volume of ether, and as much water as will cause the ethereal layer just to separate clearly, may be added to the dark green lye, which contains a heavy precipitate of reddish brown, potash salts. The ethereal layer is drawn off and the lye extracted several more times with considerable ether. The combined, brownish, ethereal solutions contain the phytol. Or, the alkaline fluid may be vigorously shaken in the flask, with very much ether and without the addition of water, enough ether being used to cause the caustic alkali to precipitate in a pulverulent form with the salts of the cleavage products and to dissolve the methyl alcohol. The supernatant liquid can then be easily decanted and the tenaceous mass of salt paste extracted a couple more times by strong shaking and stirring with ether.

Second method (that of Willstätter and Utzinger). Saponification by shaking with cold, alcoholic potash.

Cold saponification, while shaking in a machine, can be easily carried out on a large scale and spares the phytorhodin.

[3] Ann. d. Chem. 380: 162. 1911, and 382: 189. 1911.

For each gram of pheophytin, when working on a small scale, use 10 cc. of a concentrated, approximately 40 per cent lye which has been prepared from 1 l. of methyl alcohol and 600 g. of stick potash, and shake in a thick-walled flask together with quartz fragments or balls for 2–3 days till the completion of the saponification is recognized by means of the basicity test. If comparatively large quantities of pheophytin are treated, use 6 cc. of the lye for each gram, instead of 10 cc.

An apparatus manufactured by Leune, of Paris, the "Broyeur Borrel," is suitable for this purpose; this permits a flat flask, mounted upon its horizontal axis, to rotate so that its contents are subjected to the action of glass pellets and emulsified.

The saponification of the simple alphyl pheophorbides is accomplished in the same manner, though more easily since they may be finely pulverized. About two hours shaking with ten times as much alcoholic potash and the addition of 10 per cent of water are sufficient for the hydrolysis of these.

Third method.[4] Hot quick saponification, using pyridine and very much potash lye.

If it is not a question of the elaboration of large quantities of material but of the formation and separation of chlorin *e* and rhodin *g* with the best approach to a quantitative determination, then the following method, by means of which these basic cleavage products are obtained with the smallest admixture of weakly basic derivatives, deserves the preference.

6 g. of pheophytin are dissolved in 20 cc. of pyridine at 80° and the warm solution is introduced in a thin stream, while stirring with a silver rod, into the lye which has been prepared from 250 cc. of methyl alcohol with 160 g. of alcohol-purified caustic potash. This is contained in a tall, silver beaker and is kept at a gentle boil.

The boiling may be interrupted after a half minute and the beaker cooled with cold water. The saponification is complete and testing of the cleavage products shows that only traces of weak bases are present.

Fractionation of the cleavage products. First example.[5]

This is a question of the elaboration of the products of the last described saponification of 6 g. of pheophytin. In order to isolate these products in a very pure state and to avoid losses while doing so the

[4] Unpublished.

[5] Unpublished; for the method previously used see Ann. d. Chem. 354: 232. 1907.

procedure that was used for the determination of the components is modified for operations on a large scale.

The alkaline solution is washed with water in a 7 l. separatory funnel and 3 l. of ether are added. The major portion of the basic compounds is transferred to the ether by the gradual addition of 20 per cent hydrochloric acid and vigorous shaking. A portion of the rhodin may separate in the form of floccules during this procedure. They are then run off with the aqueous layer, again made alkaline with ammonia, and the residue transferred by renewed acidification to two more liters of ether in another separatory funnel.

The whole ethereal solution is extracted 8 times, using 1 l. of 3 per cent hydrochloric acid each time; the greenish blue chlorin solution is washed with ether in order to remove traces of rhodin, using 200 cc. of ether for each of two extracts. The solution is then neutralized with ammonia till its color changes to violet blue. At this point the substance is easily transferred to ether and it is quantitatively extracted by means of two extractions with 3 l. and 1 l. of ether respectively. The ether is carefully washed with only a little water. As long as this takes up only a trace of acid it remains free from chlorin, but upon repeated washing with larger quantities of water, the phytochlorin passes over abundantly, with an olive green color, into the pure water from which ether can reextract it only upon the addition of some acid; this behavior cannot be observed in the case of the ethereal solutions of previously isolated phytochlorin.

The ethereal rhodin solution still contains a small admixture of chlorin from which it is freed by two extractions, each made with 1 l. of 5 per cent hydrochloric acid. The substance is transferred from the acid liquor into 1 l. of ether which is then reextracted with 1 l. of 3 per cent hydrochloric acid. The acid portion is added to the chlorin fraction, while the ethereal layer, after it has been washed with 5 per cent hydrochloric acid, is added to the rhodin fraction.

The phytorhodin *g* can be extracted by 6 to 8 extractions, each made with 1 l. of 9 per cent hydrochloric acid; all the extracts are washed, successively or in pairs, with the same 500 cc. of ether. The ethereal mother liquor, which is a very dilute rhodin solution containing a small quantity of weakly basic chlorin, can be finally exhaustively extracted by 3 extractions, each made with 0.5 l. of 12 per cent hydrochloric acid. These are diluted to a hydrochloric acid content of 7 per cent, carefully washed with ether, and united with the main solution.

The rhodin solution is neutralized in three portions till it becomes turbid green in color and is then extracted with ether, using 6–8 l. in all. Without separating the ether each portion is neutralized still further with ammonia and again shaken vigorously.

The chlorin and rhodin solutions are finally dried with sodium sulphate and evaporated, the former to about 1.5 l. and the latter to 2.5 l. The solutions are then filtered from the material that has separated from them and are then evaporated still further, taking care that the water in the bath does not stand higher than the level of the ether in the flask, as solid crusts would otherwise adhere firmly to the walls of the flask.

The yield of beautiful crystals amounts to 1.05 g. of phytorhodin *g*; that is, almost the total quantity possible, and 2.2 g. of phytochlorin *e*; that is, 82 per cent of the theoretical.

Second example.

The first example, briefly described despite all its cumbersomeness, presents an analytical method.

In preparative work the same degree of purity of the cleavage products is not usually aimed at and the fractionations are, indeed, always carried out with greater losses in these products. The sum of the two products seldom exceeds 40 per cent of the pheophytin.

It is necessary to shorten the fractionation that has just been described in order to obtain phytochlorin in the pure lactam hydrate form. This form is dehydrated on remaining in a hydrochloric acid solution for some time, but for storage it offers the advantage of greater stability.

After removal of the phytol, the alkaline mass from 20 g. of pheophytin, for example, is greatly diluted and the chlorophyll derivatives are transferred by acidification to 12 l. of ether. The phytochlorin is sufficiently extracted by shaking 3 times with 4 per cent hydrochloric acid, using 4 l. in all, and each of the acid extracts is washed with 1–1.5 l. of ether in order to separate all the accompanying rhodin. The phytochlorin is isolated from the purified acid solutions by their approximate neutralization and extraction with ether and obtained as violet crystals of the hydrate by concentration to 0.5 l.; yield 5.2 g.

After the separation of the main portion of the chlorin it is only necessary to shake several times with 6 per cent hydrochloric acid to remove the last traces of it in order to leave the rhodin solution sufficiently pure for crystallization. After the dilute ethereal solution has

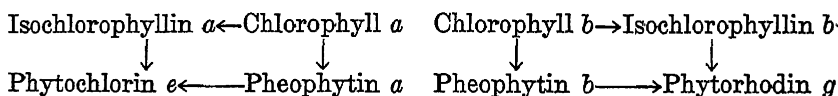
been concentrated, black lustrous prisms of rhodin separate and leave a brownish colored mother liquor; the yield amounts to 2.8 g.

In consequence of the long procedure involved in the isolation of phytochlorin *e* as described, rather long standing of the hydrochloric acid solution, and repeated transfers to hydrochloric acid, the characteristic plates of the anhydrous form customarily appear in addition to the violet crystals.

As a result of the elaboration of the cleavage products from 7 g. of ethyl pheophorbide in this manner the phytochlorin solution first produced, on concentration to 700 cc., a voluminous precipitate of 1 g. of the lustrous, black leaflets of the anhydrous modification, and then, only after further concentration, the compact, violet lustered crystals of the hydrate (1.8 g.).

2. Formation from the Chlorophyllides.

The isochlorophyllin salts *a* and *b* are formed by the hot saponification of chlorophyll and the other chlorophyllides. These salts are simply the magnesium derivatives of phytochlorin *e* and phytorhodin *g* and consequently, on acidification, they produce directly, and without any secondary products, the same compounds that were, on the other hand, obtained by the successive action of acids upon the chlorophyllides and of alkalies upon the pheophorbides:



This method for preparing chlorin and rhodin did not prove successful until recently, since saponification to the isochlorophyllin salts cannot be carried out easily with dilute chlorophyll solutions and since it is precisely saponification under mild conditions by way of the chlorophyllin series that gives the feebly basic compounds (chlorin *g*, rhodins *k* and *i*).

Willstätter and Utzinger[6] were the first to obtain phytochlorin *e* and phytorhodin *g* by the action of considerable hot barium hydroxide upon a crude chlorophyll solution and acidification of the barium salt that was thus formed, and to identify them by analysis.

Our cleavage test, which is also used for the determination of the component ratio of chlorophyll preparations, depends upon the quantitative formation of these two compounds.

[6] Ann. d. Chem. 382: 162. 1911.

By means of this decomposition chlorin *e* or rhodin *g* are obtained without any trouble; *i.e.*, without fractionation, from the pure chlorophyll components.

The elaboration of mixtures of the chlorophyll components,[7] however, requires the same separation that was found necessary in the already described preparation from pheophytin; this is simplified, though, by the fact that the formation of the weaker bases can be wholly avoided.

4 g. of methyl chlorophyllide, or 6 g. of powdered chlorophyll, are introduced, in several portions, into 100 cc. of 35 per cent pure methyl alcoholic potash lye, which is stirred and heated to boiling in a tall silver beaker. Under these conditions boiling for only a few minutes is sufficient to prevent the appearance of ester acids. After cooling, the liquid is washed with water into a separatory funnel and, after a layer of 4 l. of ether has been added, acidified. The chlorin is extracted, as above, from the ethereal layer by means of 3 per cent hydrochloric acid and pure rhodin remains in the ethereal layer after it has been washed with 5 per cent acid.

The yields are not far from the theoretical.

3. Description.

Phytochlorin e.

It appears in two modifications, as the lactam hydrate of the formula $C_{34}H_{36}O_6N_4$; *i.e.*, $(C_{31}H_{32}N_4)$ $(C(OH)_2)$ $(COOH)_2$, compact, opaque, crystalline leaves with a dull, violet luster, and as a lactam of the formula $C_{34}H_{34}O_5N_4$; *i.e.*, $(C_{31}H_{32}N_4)$ (CO) $(COOH)_2$, black lustered, approximately rectangular plates (Plate IV, Fig. 3).

It appears natural to explain the hydrated form as a tricarboxylic acid. But the appearance and disappearance of the yellow phase in the formation of phytochlorin can be best understood when considered to be a consequence of the opening and reclosing of a lactam group which is, therefore, assumed to be present in both modifications. These also show extensive agreement in their hydrochloric acid number, 3, as well as in other characteristics.

The powder of both forms is bluish black; the crystals are light green, olive green and brown by transmitted light.

[7] Unpublished.

The pure hydrate form is a stable substance. Preparations that had been dried by heat remained unaltered on storage, as did also preparations that were not dried.

The anhydrous modification can be kept unaltered only when in the condition in which its crystals were isolated from the ether. When dried it changes partly into amorphous, insoluble material, partly into weakly basic chlorin and also, finally, into a rhodin that forms a characteristic ammonium salt, soluble in ether with a green color.

Their solubilities, likewise, are not the same. The hydrate, above all, is very difficultly soluble in cold alcohol, and but slightly more so in hot alcohol; it is very difficultly soluble in acetone and almost insoluble in chloroform. In boiling glacial acetic acid it dissolves rather difficultly; in the cold, difficultly; in formic acid, easily (in both acids with a deep blue color); and, in pyridine, easily with an olive color. The anhydride form is rather easily soluble in cold glacial acetic acid; in acetone, rather difficultly, and in pyridine it is very easily soluble. It dissolves rather easily in cold alcohol and in chloroform; if a saturated alcoholic solution is warmed for a short time, crystals in the form of small, rectangular plates with rounded angles precipitate. These appear brownish red by transmitted light.

The crystallized phytochlorin is soluble with extreme difficulty in ether; ethereal solutions formed by the aid of acid or ammonia are tinted olive green.

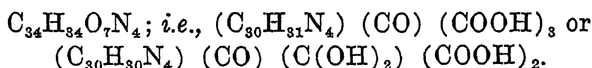
The solution in concentrated sulphuric acid is blue green; in concentrated hydrochloric acid, emerald green, almost like phytorhodin *g* dissolves in dilute hydrochloric acid but tinged greenish; on dilution, the solution becomes bluer and bluer, reaching pure blue in 2 per cent hydrochloric acid. The dilute hydrochloric acid solution becomes more violet upon the addition of a little alcohol.

In concentrated sulphuric acid chlorin changes into an amorphous substance, insoluble in ether; in hydrochloric acid it undergoes no great change, but the hydrate modification loses water when it stands in the acid. For its transformation into the lactam form it was dissolved in 20 per cent hydrochloric acid, diluted to a 4 per cent hydrochloric acid content and allowed to stand for a week; the re-isolated material crystallized from ether in the typical, black, lustrous forms of the lactam and was apparently pure; still, according to analysis the cleavage of water was not yet complete.

Phytochlorin, in both modifications, shows strongly acid properties. It is partly extracted from ether even by 0.001 per cent ammonia; completely, by 0.01 per cent; in traces by 0.01 per cent disodium phosphate and rather easily by 0.02 per cent. The solutions in these most dilute alkalies show a characteristic, violet color although the substance dissolves with an olive green color in ammonia or other alkalies of somewhat greater concentrations.

A characteristic derivative of phytochlorin is its trimethyl ester, $(C_{34}H_{33}O_3N_4)$ $(OCH_3)_3$, which is formed by grinding its potassium salt with methyl sulphate. It is rather easily soluble in alcohol and ether and crystallizes in steel blue, matted prisms with a melting point of 188–190°. Its hydrochloric acid number is 7 and it forms phytochlorin again with alcoholic potash.

Phytorhodin g.



$C_{34}H_{34}O_7N_4$; *i.e.*, $(C_{30}H_{31}N_4)$ (CO) $(COOH)_3$ or
$(C_{30}H_{30}N_4)$ (CO) $(C(OH)_2)$ $(COOH)_2$.

It crystallizes from ether in large, compact, six-sided prisms with oblique ends (Table IV, Fig. 4); they exhibit a dark red, almost black, metallic luster. The solutions in ether, alcohol and glacial acetic acid are deep red with a bluish tint and possess a very weak, dark red fluorescence.

In its crystalline condition the substance is insoluble in ether and in chloroform; it is quite easily soluble in alcohol and glacial acetic acid. Rhodin *g* dissolves very easily in pyridine, it dissolves easily in formic acid with an emerald green color just as it does in concentrated hydrochloric acid. Although its hydrochloric acid number is 9, the solid substance requires 17–20 per cent hydrochloric acid to dissolve it, if this is used without ether.

Phytorhodin *g* is a strongly acid compound; it is easily extracted from an ethereal solution even by 0.001 per cent ammonia, and completely so by 0.02 per cent disodium phosphate, with a reddish green color.

The salts of phytorhodin with potassium or caesium hydroxide are tertiary. They are converted by methyl sulphate into the trimethyl ester which crystallizes in black lustered, rectangular and trapezium-shaped plates, melting at 207–210°. Its hydrochloric acid number is 13.

The absorption spectra[8] (Plate X, Chapter XXV).

A comparative investigation of the absorption spectra shows strikingly the relationship of the two chlorophyll components, and their magnesium-free derivatives, to their cleavage products, phytochlorin *e* and phytorhodin *g*.

Phytochlorin *e*, in ether, shows a spectrum composed of five sharply defined bands; this spectrum is extremely similar to that of the pheophytin component *a* except that the weak bands, II and VII, of the pheophorbide spectrum are absent, or more exactly, they are perceptible as faint shadows only. The remaining five bands are arranged not only in the same sequence according to their intensities: I, V, IV, II, III, but also agree in intensity and breadth and almost exactly in

SOLUTION OF 0.030 G. LACTAM HYDRATE IN 1 L. ETHER.
(About 1/1000 mole in 20 l.)

Depth of layer in mm.	2.5	10	20	40
Band I	672— —661	678—655	679—652 | 635	683—648 . 632
" II	—	616 | 602	617 . . 603	617 . . . 603
" III	—	—	563 | 553	564 . 553
" IV	535 . 527	535 . . 527	535 . . . 527	537— —527
" V	508 . . 492	509 . . . 491	510— —490	512—488
End absorption	411—	427—	432—	437—

position with the corresponding bands of the pheophorbide. The agreement is striking because photochlorin *e* is not the carboxylic acid that forms the nucleus of the esters, pheophytin and methyl pheophorbide.

Phytorhodin presents a beautiful spectrum of still simpler structure; the strongest absorption band of the visible region lies, as is usual in the chlorophyll derivatives, in the red but it is close to the transition to the orange. A strong, double band lies in the green and, in addition, a fourth weaker band is present in the orange. The arrangement of the bands according to their intensity is accordingly: end absorption, I, IV, III, II.

The relatively simple spectrum of photorhodin is very closely related to the much more complicated spectrum of pheophorbide *b*. The only deviations are the absence here of band VI of the *b* component, which lies upon the Fraunhofer line F, and the merging of the char-

[8] Paper XVII.

acteristic, three-branched absorption in the green, without a change of its position, into a double band.

The differences on comparison with phytochlorin *e* are, of course, much more important; in the case of rhodin the two bands in the red and orange are displaced towards the violet; instead of three bands being distributed over the entire green region rhodin shows but two strong bands.

Formation of complex potassium compounds.[9]

The cleavage products of chlorophyll can form compounds with the alkaline hydroxides and these remind us to an astonishing degree of the natural magnesium compounds. The alkali salts of chlorin and rhodin, in alcoholic solution, reproduce very closely the absorption spectra of the magnesium-free chlorophyll derivatives from which they

SOLUTION OF 0.030 G. RHODIN IN 1 L. ETHER.

(About $^1/_{1000}$ mole in 20 l.)

Depth of layer in mm.	2.5	10	20	40
Band I	659 .. 649	662—649	667—641	673—636
" II	602 | 595	603 . 594	607 ... 592	608— —590
" III	568 | 555	569 . 554	570 ... 553	576— —552
" IV	537 . 517	536 .. 518	539— —516	541—509
End absorption	446—	451—	455—	459—

have been derived; namely, the chlorin alkali agrees with the *a* component of pheophytin and the rhodin alkali with the *b* component. If, on the other hand, phytochlorin and phytorhodin are dissolved in very concentrated alcoholic potash lye or, better still, if their solutions in 30 per cent methyl alcoholic potash lye are evaporated to a content of 45 per cent potassium hydroxide, the color changes completely to an intensive chlorophyll green; in one case, to the blue green of the *a* component and, in the other case, to the yellow green of the second component (*b*).

The potassium compounds that are thus formed are compared with the *a* and *b* isochlorophyllin salts. These, when dissolved in highly concentrated, methyl alcoholic potash lye, agree in their absorption

[9] Ann. d. Chem. 385: 180. 1911.

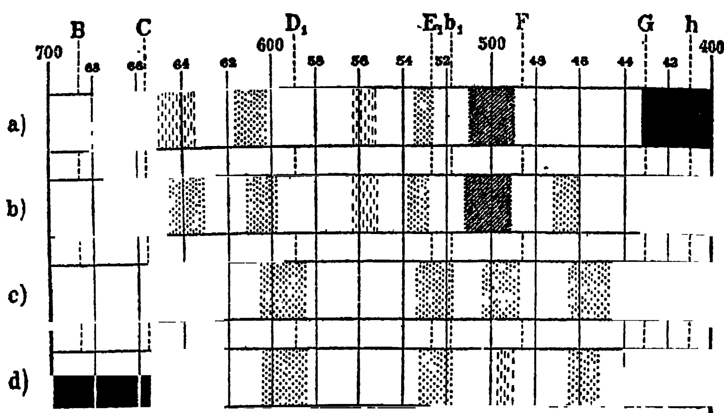


Fig. 11.

$^1/_{1000}$ mol. in 20 l.; depth of layer 20 mm.

a. Phytochlorin *e* in ether;
b. Phytochlorin *e* in 5 per cent methyl alcoholic potash;
c. Phytochlorin *e* in 45 per cent methyl alcoholic potash;
d. Isochlorophyllin *a* in 44 per cent methyl alcoholic potash.

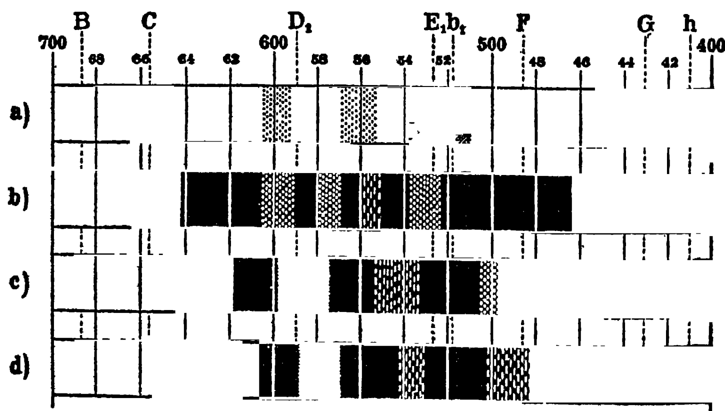


Fig. 12.

$^1/_{1000}$ mol. in 20 l.; depth of layer 20 mm.

a. Phytorhodin *g* in ether;
b. Phytorhodin *g* in 5 per cent methyl alcoholic potash;
c. Phytorhodin *g* in 48 per cent methyl alcoholic potash;
d. Isochlorophyllin *b* in 45 per cent methyl alcoholic potash.

phenomenon with chlorin and rhodin, dissolved in the same media and, indeed, so completely so that it must be assumed that the uni-

valent alkali metal is bound analogously to the magnesium in chlorophyll.

This metallic complex, the most unstable one that the chlorophyll derivatives form, undergoes dissociation even when the alkaline mass is diluted with alcohol.

Figures 11 and 12 display the absorption spectra of chlorin and rhodin in juxtaposition with those of their normal and their complex alkali compounds and with those of the corresponding iso-chlorophyllins.

4. The Weakly Basic Phytochlorins and Phytorhodins.

Phytochlorin f.[10]

If chlorophyll undergoes a change such that the brown phase is lost, as upon the standing of many of its extracts, pheophytin hydrolysis frequently produces phytochlorin *f*. This was first isolated by Willstätter and Hocheder[11] in the elaboration of Ulva lactuca. The appearance of this cleavage product is not specific for extracts of the plant just named; on the contrary, the same transformation of chlorophyll takes place in other extracts and it is often observed, especially in the case of stinging nettles. Phytochlorin *f* has been obtained from impure extracts only, although not in any experiment in which the leaves have been previously treated with benzol and petroleum ether. Likewise, in pure petroleum ether solutions the chlorophyll is not altered in such a manner that the decomposition produces phytochlorin *f*. Phytochlorin *g*, which is similar in basicity but entirely different in its other properties, is formed instead.

Phytochlorin *f* has not yet been obtained from chlorophyllin.

Pheophytin preparations that had been obtained from stinging nettle extracts that had stood for some time after their filtration from the meal previous to acidification often served as initial material for the preparation of this phytochlorin. After saponification the mixture of cleavage products was fractionated with hydrochloric acid in order to separate chlorin *f* from the abundant admixture of phytochlorin *e* as well as from phytorhodin *g*. The phytochlorin *f* was extracted with 11 per cent hydrochloric acid, freed from phytorhodins *i* and *k* by washing with ether, diluted to a 7–8 per cent hydrochloric acid content and then again extracted with ether. Upon concentration of the

10 Paper XVI.

11 Ann. d. Chem. 354: 237. 1907.

ethereal solution the chlorin separated as violet black crystals. These are rhombic plates which have an olive brown color by transmitted light (Table IV, Fig. 5). The preparation, once isolated, crystallizes from ether in beautiful, crystalline aggregates of radially arranged prisms.

Phytochlorin *f*, $(C_{31}H_{32}N_4)$ (CO) $(COOH)_2$, is an isomer of the anhydrous form of phytochlorin *e*. Its behavior towards ammonia gas, of which it takes up only half as much as does chlorin *e*, appears to indicate that a dilactammonohydratemonocarboxylic acid occurs in it.

Chlorin *f* is soluble with extreme difficulty in all the usual solvents. It is easily soluble in pyridine as well as in formic acid, of course with the formation of salts and, in fact, with a pure blue color in the latter. The ethereal solution prepared from the ammonium salt has a beautiful green color and is much less tinged with olive than is the solution of chlorin *e*. Its color in 11 per cent hydrochloric acid (the HCl number is more exactly 10) is a pure blue while phytochlorin *e* in acid of this concentration is greenish blue and chlorin *g* has a bluish green color. Phytochlorin *f* is extracted from its ethereal solution by ammonia of the same concentration as extracts chlorin *e* but it is distinguished from the latter by the pure green color of the extract.

Chlorin *f* forms a beautifully crystallized caesium salt which forms with methyl sulphate an ester that crystallizes in plates or prisms.

Phytochlorin *f*, on decomposition with hot concentrated alkali, produces rhodoporphyrin and then pyrroporphyrin (see Chapter XX, section 4).

Phytochlorin g.[12]

This is a cleavage product that very frequently appears when chlorophyll is decomposed. It is similar to phytochlorin *f* in its basicity. Its composition must be very much like that of phytochlorins *e* and *f*, and only a difference of H_2O can be involved in the empirical formula. Since this compound is easily altered its analysis has not yet been successful, but it was considered important to observe its mode of formation and to fix its characteristics.

Phytochlorin *g* is formed from undamaged chlorophyll:

1. By the saponification of pheophytin when its ethereal solution is treated with alcoholic lye.

[12] Paper XVI.

2. By cleavage with acid of chlorophyllin salt that has been obtained by means of cold alcoholic lye. The isolated chlorophyll component *a*, by solution in alcohol or pyridine and introduction into cold alcoholic lye, also produces a chlorophyllin which, on decomposition with mineral acid, forms phytochlorin *g*.

Phytochlorin *g* is also formed from altered chlorophyll. The *a* component in a petroleum ether solution that contains alcohol changes, on standing, in such a way that the hydrolysis of its pheophytin produces this chlorin. It is produced in the same manner from the easily soluble derivatives of ethyl chlorophyllide that are formed in alcoholic solutions.

In order to obtain it Willstätter and Utzinger saponified the crude, petroleum ether solution of chlorophyll component *a* with alcoholic potash and acidified the alkaline fluid with hydrochloric acid. The cleavage product was then extracted with ether and freed from some admixed chlorin *e* by means of 6 per cent hydrochloric acid.

The ethereal solution of phytochlorin *g* that remains is olive green, similar to chlorin *e*, while chlorin *f* is pure green. On standing, the solution becomes brown to brownish red as a result of decomposition. The hydrochloric acid number is 10–11.

Upon evaporation the color of the ethereal solution changes easily to a brownish-tinged red. The color change on evaporation and heating for a short time with alcohol is a characteristic of phytochlorin *g*. A very beautiful, but very unstable, compound which is red in indifferent solvents and similar to the phytorhodins is formed here. It is strongly basic so that it is extracted from ether even by 1 per cent hydrochloric acid, and even by slightly acidified water, with a light sea-green color; furthermore, it is extracted from ether by aqueous alcohol. It dissolves in dilute ammonia with a red color.

Phytochlorin *g*, when heated with alcoholic potash to 140–150°, yields a porphyrin that is closely related to glaucoporphyrin; at 225–230°, pure pyrroporphyrin.

The Phytorhodins i and k.[18]

There are formed from chlorophyll *b*, analogously to the case of the *a* series, by cold saponification and the cleavage of magnesium or by allomerization and subsequent cleavage in different ways, instead of

[18] Papers XVI and XXII.

phytorhodin *g*, two more weakly basic products, phytorhodins *i* and *k*. These can be separated only as a result of a small difference in their basic properties by a careful fractionation according to the method of Willstätter and Mieg, using 14 per cent hydrochloric acid. The hydrochloric acid number of rhodin *k* is, namely, 14–14½; of *i*, 15–16.

Many methods of formation give mixtures of rhodins *k* and *i*, in which *k* frequently predominates.

Considerable rhodin *k*, in addition to a little *i*, is formed in the saponification of pheophytin or methyl pheophorbide *b*; suitably of a dilute ethereal solution by means of cold methyl alcoholic potash.

Chlorophyll or methyl chlorophyllide *b*, on saponification in cold lye, produces, about half, strongly basic rhodin and, about half, the two more feebly basic rhodins.

Allomerization of chlorophyll *b* furnishes a rich supply of initial material for the feebly basic rhodins. When allomerized preparations are saponified in alcoholic solution with lye a little rhodin *i* is obtained besides the predominant product, *k*. The proportion of rhodin *k* was especially large when the allomerized chlorophyll was first split with acid and then with alkali.

In the case of the allomerization of chlorophyll *b* in petroleum ether, on the other hand, solutions were formed that by the action of methyl alcoholic potash and subsequent acidification produced abundant quantities of the two weakly basic derivatives.

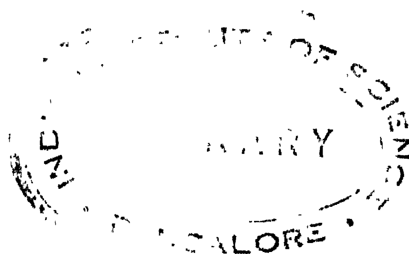
The fractionation of the cleavage products from an ethereal solution by means of hydrochloric acid is tedious on account of the admixture of stronger bases, and in consequence of the small difference in basicity the strongest bases are removed by frequently repeated extractions with a little 13 per cent hydrochloric acid; 14–14½ per cent acid then extracts rhodin *k*, and 17 per cent, phytorhodin *i*. The two cleavage products are again transferred to ether by diluting their hydrochloric acid extracts after these have been washed with ether, and completely freed from stronger bases by shaking with 12½, or with 14½, per cent hydrochloric acid. The pure rhodins crystallize from the ethereal solutions, after they have been washed with water and concentrated, in poorly defined plates.

Phytorhodins *k* and *i* coincide in their composition, $C_{34}H_{32}O_6N_4$. They appear to differ from phytorhodin *g* by the absence of 1 mol. of water.

The solubility of the phytorhodins in ether is very slight; they are difficultly soluble in cold alcohol; rather easily soluble in hot alcohol. Their ethereal solutions are less red than those of phytorhodin *g* and they have a more brownish tint. In the case of phytorhodin *k* the hue is a little more red than with *i*.

They dissolve in 20 per cent hydrochloric acid with a light green color; in ammonia, with a brown color. In dilute methyl alcoholic potash *k* gives a beautiful red solution; *i*, a yellow solution.

On the addition of a few drops of concentrated nitric acid, the alcoholic solution of rhodin *k* turns deep blue; that of *i* turns brownish red.



XVII. PHYTOL.

1. Preparation and Quantitative Determination.

Pheophytin, according to Willstätter and Hocheder,[1] is easily saponified by alcoholic potash, just as any other wax is; chlorophyll behaves similarly.

Although the composition of the acid components; *i.e.*, of the sensitive, nitrogenous cleavage products, is extraordinarily dependent upon the conditions of saponification, these do not, in general, have any influence at all upon the composition of the alcohol that is split off, and they have but little influence on the yield of the same. There is only one method by which phytol is obtained in an altered form; *i.e.*, chiefly as phytyl methyl ether. This method is the methanolysis of pheophytin with hydrochloric acid in methyl alcohol.[2] After the isolation of chlorophyll had been accomplished it was possible to solve the question whether phytol as such constitutes a part of the molecule of the pigment; it was shown that the unsaturated alcohol is not possibly formed from a saturated glycol as an immediate result of the conditions under which the pheophytin is saponified because Willstätter and Hug[3] split off phytol from chlorophyll under gentle conditions of alcoholysis by means of chlorophyllase and isolated it with special caution. The iodine number indicated that the alcohol that was thus split off was pure phytol.

Two methods have been given in the first section of Chapter XVI for preparing phytol by the saponification of pheophytin with alcoholic lye; that carried out with heat and that carried out in the cold. The extraction of phytol from the product of the reaction has already been described there. The ethereal solutions are colored brown at first by a small admixture of nitrogenous substances which may be best removed by successive treatments with alkali, hydrochloric acid and animal charcoal. The ethereal solution is first shaken with a very

[1] Ann. d. Chem. 354: 240. 1907.

[2] Chapter XV, section 3.

[3] Chapter IX, section 1.

dilute lye and then assiduously a number of times with a little concentrated hydrochloric acid, which turns blue green in color. The ether is then washed many times with considerable water and concentrated; for example, in working up 200 g. of pheophytin, to 1.5 l. Finally, the last colored impurities are removed by shaking in a machine for several hours with good, pure animal charcoal. The ethereal solution is dried with sodium sulphate, then concentrated and completely evaporated in vacuo. The residue from the evaporation is heated, with an immersed capillary, for 3/4 of an hour in a vacuum at 90° C. in order to remove the last traces of solvent.

The animal charcoal retains some phytol. It pays to collect this charcoal and extract it separately with ether, but only a portion of the absorbed phytol (namely, 0.1–0.5 per cent of the pheophytin) is recoverable.

In connection with the absorption of phytol by animal charcoal the following experiment is of interest: 5.7 g. of phytol were shaken for 5 hours in 500 cc. of ether with 11 g. of dried animal charcoal; the animal charcoal took up 1.38 g. of phytol. Upon boiling for 6 hours with 500 cc. of ether only 0.54 g. of phytol were given up again.

The isolation of phytol (of which several kilograms were obtained) is almost quantitative. The yield amounts to almost a third of the pheophytin.

The theoretical phytol number; *i.e.*, the calculated phytol content, expressed in per cent, amounts to:

for pheophytin *a*, 33.7; for pheophytin *b*, 33.2;
for the mixture having the component ratio 2.5, 33.56;
for chlorophyll " " " " 2.5, 32.66.

The quantitative determination[4] of phytol has played an important rôle in the comparative investigation of the chlorophyll from different plants. Essentially the same procedure serves for this as for the preparation of phytol on a large scale.

0.3 to 1 g. (usually about 0.5 g.) of pheophytin or chlorophyll are used for the quantitative determination. The finely ground material is boiled for 2 hours on a water bath with 24 per cent methyl alcoholic potash (5–6 cc. for 1 g.). This is done in a test-tube-like vessel (30–40 cc. content) having a constricted neck and a ground-on cooling tube when small quantities are used, and in a round flask with a ground-on

[4] Ann. d. Chem. 371: 18. 1909 and 378: 31. 1910.

condenser when somewhat larger quantities of pigment are used. The phytol is then extracted 5 or 6 times with ether. This is most conveniently done in the same vessel without the addition of water. The mass, which becomes viscous, is well stirred and shaken with the ether each time and this is then simply decanted. The alkaline fluid, of course, may also be washed with water into a separatory funnel and there extracted with ether, but this method is time-consuming because emulsions often appear. The united ethereal solutions are washed with water alone several times and dried with ignited sodium sulphate. They are then shaken with pure, but not specially dried, animal charcoal for 15 minutes, using 0.1 g. of animal charcoal for a concentration of 1 g. of pheophytin in 200 cc. of ether. The same quantity of charcoal and the same concentration of ethereal solution are always used in order to make the loss of phytol by adsorption on the charcoal as uniform as possible.

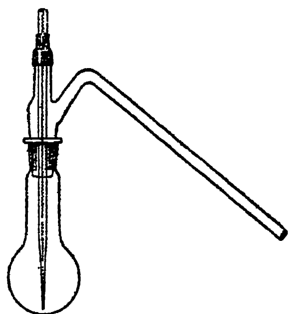


Fig. 13.

The solution is filtered again and evaporated on a water bath in a distilling flask, using a capillary as fine as a hair to prevent bumping; finally, the residue is washed with a little ether into a light, tared, 15 cc. weighing bulb with a long neck (in order to prevent spattering). This also has a ground-on cap (see Fig. 13). The ether is expelled by heating in vacuo at 90° for 45 minutes till the weight is constant, an immersed capillary being used, and the small phytol flask is then weighed upon an analytical balance.

The fat content of Kahlbaum's ether usually caused an error of about + 0.2 per cent when 0.5 g. of pheophytin was used and this compensated well for the unavoidable loss of phytol in the isolation.

This was because every 200 cc. of ether gave a fatty residue weighing 0.0033–0.0028 g.; in carrying out all the purifying operations such as are involved in a phytol determination, however, the residue amounted to 0.0012 g. But since many commercial samples of ether have a much larger residue, it is preferable to employ only freshly purified and distilled ether for the determinations.

In repeated determinations, the values differed very little, as the following examples show.

Two determinations on a pheophytin preparation from Heracleum gave the phytol number 29.5 and 29.6 (0.5201 g. gave 0.1537 g.; 0.5315 g. gave 0.1573 g.); a preparation that had been more thoroughly alcoholized gave the phytol numbers 23.0 and 23.2 (0.5433 g. gave 0.1252 g.; 0.3731 g. gave 0.0857 g.); another, the values 25.6 and 25.7 (0.4213 g. gave 0.1080 g.; 0.3102 g. gave 0.0798 g.).

2. Description.[5]

Phytol is an unsaturated, primary alcohol of the aliphatic series, having the formula $C_{20}H_{39}OH$. Its carbon chain is branched.

It is a colorless, rather heavy oil, which mixes with all the ordinary organic solvents. It boils without decomposition under diminished pressure, and it is best purified by distillation[6] in high vacuum.

Its boiling point under 0.03–0.04 mm. pressure is 145°; under 9–11 mm., 203–204°.

$$D_4^0 = 0.864, \quad d_4^{20} = 0.852; \quad n_D^{20} = 1.46380.$$

Phytol is auto-oxidizable and combines easily with ozone; it takes up a molecule of bromine (1.05 mol. found instead of 1 mol.) and gives an iodine number (90.5 and 91.2 found, instead of 85.5 obtained by calculation) which closely agrees with this.

The sodium salt of phytol is easily soluble in ether. With phenyl cyanate, phytol forms a crystallizable urethane, which has the melting point 26–29°. It unites with phthalic acid anhydride to form an ester acid the characteristic silver salt of which is easily soluble in ether, as well as in benzol, and crystallizes in prisms with a melting point of 119°.

By treatment with hydrogen in the presence of platinum the unsaturated alcohol can be hydrogenated to a saturated alcohol. Dihydrophytol ($C_{20}H_{42}O$) is an oil that is miscible with organic solvents and it has a boiling point of 201.5–202° under 9.5 mm. pressure; $D_4^0 = 0.849$, $n_D^{20} = 1.45213$.

[5] Papers III and XII.

[6] A duck-shaped, distilling flask (Fig. 14) is used for the distillation of high boiling substances. It should not be too large and still it should not permit any spattering over.

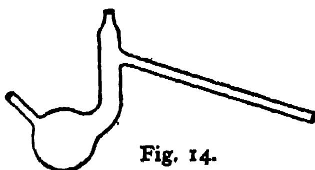


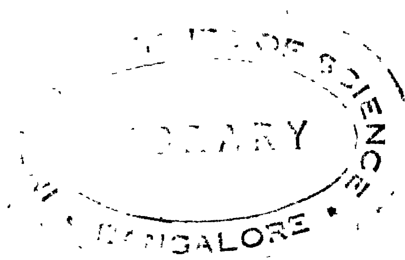
Fig. 14.

Phytannic acid ($C_{20}H_{40}O_2$) is formed by heating dihydrophytol with soda lime. This is an oil of high viscosity with a boiling point of 221° under 7.5 mm. pressure.

The parent hydrocarbon of phytol, with the formula $C_{20}H_{42}$, phytane, appears as a secondary product in the hydrogenation of the unsaturated alcohol. It is an easily mobile fluid, slightly soluble in methyl alcohol and in glacial acetic acid. Its boiling point is 169.5° under 9.5 mm. pressure.

When phytol is oxidized with chromic acid or if its ozonide is boiled, there is a cleavage of the hydrocarbon chain between the third and fourth carbon atoms. In this way the ketone, $C_{17}H_{34}O$, is formed. It is an oil, with a boiling point of 175° (at 11 mm.) and $d_4^0 = 0.844$, which is miscible with ordinary solvents. The formula, $C_{15}H_{30}O$, first assumed for this ketone, has been found to be erroneous. Checking of the work that had been published[7] showed that the same ketone, $C_{17}H_{34}O$, in a more or less pure state, is always obtained whether crude or distilled phytol is used. This fact disproved the assumption of Willstätter, Mayer and Hüni,[8] that the double linkage shifts when phytol is distilled.

[7] O. Schuppli. Beitrage zum Abbau des Phytols. Thesis. Zürich. 1912.
[8] Paper XII.



XVIII. THE CHLOROPHYLLIN SALTS.

Disclosures that helped to reveal the composition of chlorophyll were first successfully obtained from the chlorophyllins.[1] Their acid nature made purification with chemical reagents possible; *i.e.*, their transfer from an ethereal solution into disodium phosphate, behaving as an alkali, and their liberation from this solution by means of monosodium phosphate, acting as an acid. Having been purified to some extent in this way the chlorophyllins caused a recognition of the significance of magnesium in the composition of chlorophyll.

When alkalies act upon the two chlorophyll components the easily saponifiable phytyl- and the difficultly saponifiable methyl-ester groups are hydrolyzed. The chlorophyllins that are formed are, as magnesium-containing free carboxylic acids, very easily decomposed; they are consequently investigated and used for purposes of systematic decomposition in the form of their salts.

A still further, unavoidable change takes place in this reaction with alkalies; namely, a transformation of lactam groups, which proceeds in one direction in the case of saponification with heat, in another direction in the case of saponification under very mild conditions in the cold, and often in both directions at once. (See p. 24 and those following.)

It was only after this dependence of the products of saponification upon conditions had been thoroughly recognized in an investigation of Willstätter and Utzinger[2] that the differentiation between, and the investigation of, each pair of pure chlorophyllins of the *a* and *b* series was successful.

1. Rapid saponification with the application of heat leads to the isochlorophyllins *a* and *b*. They had to receive the name of iso-compounds, because it was only possible to prepare them much later than the products of the cold saponification; namely, only at a time when pure alphyl chlorophyllides had become available. It is precisely from the isochlorophyllins that the most important derivatives of the

[1] Paper II.
[2] Paper XVI.

chlorophyll components are derived simply by elimination of the magnesium.

Thus, isochlorophyllin *a* produces phytochlorin *e* when its magnesium is eliminated by means of acid; isochlorophyllin *b*, in the same manner, produces phytorhodin *g*.

The decomposition of both isochlorophyllins with alkalics at a higher temperature leads to phyllophyllin.

The isochlorophyllin salts are distinguished by a fluorescence similar to that of chlorophyll, while the salts of the ether series, the chlorophyllin salts, do not fluoresce.

2. The saponification in the cold can be so conducted that the pure chlorophyllins *a* and *b* are obtained. On decomposition with acids they produce the weakly basic, easily alterable phytochlorin *g* and weakly basic phytorhodins, compounds that have only attained secondary importance in connection with the systematic decomposition of chlorophyll.

But the chlorophyllin salts themselves are valuable as initial material for the preparation of pyrrophyllin. In the case of saponification in the cold, the formation of iso-compounds always took place incidentally to an annoying degree. Therefore a new method, which makes it possible to produce the chlorophyllin alone in a pure state, is useful. This consists in first allomerizing the chlorophyll by allowing it to stand in a weakly alkaline, alcoholic solution; that is, in altering its lactam groups, and then in its gentle saponification in the cold. The only difficulty that then remains to be considered is that in this careful saponification the chlorophyllin easily retains the methoxyl group.

1. Hot Saponification.[3]

Hot saponification, which opened up new possibilities of obtaining products by systematic decomposition, could not be carried out with the diluted extracts of chlorophyll; consequently it could only be employed when isolated chlorophyllide or chlorophyll was available for that purpose.

Mention was made in Chapter XVI, section 2 of the formation of isochlorophyllin salts, as considered from the viewpoint of the preparation of phytochlorin *e* and phytorhodin *g* from them. In order to employ the potassium salts for the preparation of the phyllins (phyllo-

[3] Unpublished.

phyllin) and the porphyrins, for which purpose they form an excellent initial material, they are obtained in a similar manner without separating them from the alkaline solutions.

10 g. of methyl chlorophyllide $(a+b)$, or just as well, 15 g. of pure, or crude, chlorophyll, are powdered and introduced in 2 g. portions into 250 cc. of boiling, 35 per cent, methyl alcoholic potash. The lye has been previously prepared in a silver bottle, which is provided with a ground silver stopper, by dissolving 350 g. of potash that has been purified by means of alcohol in 650 g. of methyl alcohol.

In order to avoid contamination from the zinc of glass vessels the potash lye is placed for the saponification in a silver crucible of 600 cc. capacity, this being then inserted in an autoclave, and a worm condenser, made of lead, is placed around the outside of the upper rim of the beaker in order to condense the vapor of methyl alcohol. When the material is introduced the contents are vigorously stirred by means of a silver spatula and care is taken that nothing sticks to the walls. Gentle boiling, without evaporation of the methyl alcohol, is continued for 5 minutes. The isochlorophyllin salt solution that is formed is greenish black and upon dilution turns a deep green color with a beautiful fluorescence.

The product of the saponification contains the two iso-potassium compounds exclusively; on acidification, extraction with ether, and fractionation only strongly basic derivatives are obtained from it. After thorough agitation with 9 per cent hydrochloric acid an extract made with 12 per cent acid, when transferred into ether, gives a pure red solution.

Pure Isochlorophyllin Potash a.

has been isolated as such by Willstätter and Forsén. One gram portions of pure methylchlorophyllide *a* were treated by having 25 cc. of boiling, concentrated methyl alcoholic potash poured over them. The yellow phase appears at once and disappears immediately. It was difficult to obtain the product of the saponification entirely free from methoxyl; in order to attain this, it was heated for 5 minutes to a gentle boil, 2–3 cc. of water were then added and the boiling was continued several more minutes. Two such portions were united and diluted with water to 200 cc. A small linen bag, filled with potassium chloride, was then immersed in this solution until all the isochlorophyllin salt was precipitated in the form of large, light green floccules

and the fluid was almost decolorized. The mother liquor was decanted and the product collected with a little talc. After drying and powdering, the potassium compound was extracted from this by 200 cc. of boiling methyl alcohol. The blue, strongly fluorescent solution was quickly concentrated in vacuo to 10 cc., 100 cc. of absolute ethyl alcohol were added and the liquor was concentrated to half its volume in order to completely remove the methyl alcohol. On standing 2 hours in a freezing mixture the dark blue, large grained, potash compound separates almost completely (1.8 g.). It still contains some admixed inorganic matter and is only freed from chlorides by repeated reprecipitation from methyl alcohol by means of ethyl alcohol. The material is shaken with 100 cc. of cold methyl alcohol, letting a portion, weighing 0.2 g., remain undissolved. The solution is again evaporated to a very small volume under diminished pressure and mixed with 100 cc. of ethyl alcohol; 0.9 g. of beautiful potassium isochlorophyllin then separates as a dark blue powder when this stands in an ice-box.

The salt is free from methoxyl and contains 16 per cent of potassium and 3 per cent of magnesium, corresponding to the formula $(C_{31}H_{31}N_4Mg)(COOK)_3$, or $(C_{31}H_{29}N_4Mg)(COK)(COOK)_2$. It dissolves rather easily in methyl alcohol with a greenish blue color; with difficulty in ethyl alcohol and other organic solvents, either hot or cold. It is easily soluble in water.

Willstätter and Fischer obtained pure potassium isochlorophyllin *b* in solution by introducing 0.3–0.8 g. of methyl chlorophyllide *b*, dissolved in 1–2.5 cc. of pyridine, into 3–8 cc. of boiling methyl alcoholic potash and heating to complete saponification for 5 more minutes over a small flame, without considerable concentration.

2. Cold Saponification.

As long as no initial material other than crude chlorophyll solutions existed for the preparation of chlorophyllin salts, saponification in the cold was carried out in the following manner.[4]

The percolates, or double extracts, were poured, in considerable charges, into large, stoneware pots, provided with well ground-on covers, from which pots the potassium salts, after the mother liquor was drained off, could be scraped off conveniently by means of strong silver spatulas. The inner wall of the cylinder had been made smooth. The vessel had a tube 10 cm. above the bottom.

[4] Ann. d. Chem. 358: 215. 1907.

The saponification was brought about by means of 20 cc. of concentrated (28 per cent) methyl alcoholic potash per kilogram of plant meal. The extract was mixed with the lye while strongly agitating. The end of the saponification could be recognized by the separation of a pure yellow, ethereal layer when a sample was shaken with water and ether. The reaction, which is completed almost instantaneously in the case of small test samples with an excess of alkali, required several hours here. During this time no chlorophyllin at all precipitated but a large amount of a dark colored, resinous precipitate that was almost free from chlorophyll material was formed and the solution had to be transferred from this into another decanting jar. It then stood 7–10 days, while well closed, in order that the chlorophyllin might be deposited.

The tenacious deposit of potassium chlorophyllin was triturated and washed with absolute alcohol and dried in a desiccator. It then formed a blue-black, hard, hygroscopic mass.

The yield from 100 kg. of stinging nettles amounted to 306–321 g. of potassium salt which, in most cases, was 47–56 per cent pure.

The alcoholic mother liquor from the salt, which still contained much saponified chlorophyllin, could be utilized for the elaboration from it of certain, ether-soluble, chlorophyllin salts.[5] The sodium, calcium and magnesium salts are, in the presence of the many other substances contained in the mother liquor, easily soluble in ether.

The mother liquor, in 15 to 20 l. portions, is diluted in glass carboys with 1½ times its volume of water and the calcium salt precipitated by the addition of 1 kg. of calcium chloride to the solution. This is done while vigorously shaking; all the floccules that separate then agglomerate, in most cases, into a semi-solid sticky mass, which clings fast to the walls of the vessel. This is still more easily brought about by using lukewarm water for the dilution of the chlorophyllin solution. After the lye has been removed the calcium salt is dissolved from the carboy by means of 2–3 l. of ether; the ethereal solution is washed several times with water and dried with sodium sulphate. The salt can now either be precipitated by means of petroleum ether or it can be separated by means of alcohol after the ethereal solution has been strongly concentrated. The alcohol, on being concentrated, yields a mother-liquor portion that is still very impure.

[5] Ann. d. Chem. 358: 218. 1907.

The mother liquor from the potassium chlorophyllin (189 g.) from 66 kg. of stinging nettles produced 620 g. of calcium chlorophyllin. This was obtained by precipitation from the ethereal solution by means of alcohol; its color was equivalent to 97 g. of crystallized chlorophyll.

New Methods.[6]

The employment of purified chlorophyll, or even of chlorophyll in the easily available form of our so-called crude chlorophyll or in that of a crude chlorophyll-talc mixture, instead of extracts, has the advantage that the purity of the chlorophyllin salt is much increased.

A second modification is even more important. When an alcoholic solution is treated with alkali at ordinary temperatures, the saponification always takes place in both possible directions; that of the formation of chlorophyllin salt and that in which iso-chlorophyllin salt is formed. Consequently we first allomerize the chlorophyll. The component *a* then no longer produces the iso-compound under any condition. This does not apply to *b*. Even after allomerization the *b* component gives the various chlorophyllin salts that are possible on hydrolysis in a cold solution and, on saponification with heat, it even gives, in addition, a large portion of the isochlorophyllin salt that corresponds to phytorhodin *g*. But if the allomerized chlorophyll is transferred into an ethereal solution and methyl alcoholic potash is then allowed to act upon it, the chlorophyllin salts that are formed are free from isopotassium compounds, that is, only the magnesium compounds of the feebly basic phytorhodins *k* and *i* (besides those of phytochlorin *g*) are formed. By the smooth formation of these chlorophyllins the yield of potassium salts *a* and *b* is also increased, because they are more difficultly soluble than the corresponding isopotassium salts.

Potassium chlorophyllin, obtained by the saponification of an ethereal solution of allomerized chlorophyll is the most suitable initial material for the production of pyrrophyllin.

5 kgs. of stinging nettle meal, in layers 3 cm. thick, were extracted in two stoneware suction filters with 15 l. of 80 per cent acetone, as described in Chapter III, section 2. The extract (9.1 l.), in one example, contained 41 g. of chlorophyll; it was mixed with 200 g. of talc and then while shaking, with 4 l. of water, so that the liquor now consisted of only a 55 per cent acetone solution and, on standing for a

[6] Unpublished.

short time, deposited almost all the green and yellow pigment. This was separated from the pale yellowish green mother liquor by filtration upon a suction filter through a little talc, washed with a liter of 65 per cent acetone, after that with a liter of 65 per cent alcohol, and then sucked as dry as possible.

The almost dry, greenish black talc is now extracted in a powder flask by shaking with 3 l. of 95 per cent alcohol and is washed with another equal portion while upon the suction filter. By employing absolute alcohol 3–4 l. are sufficient for the extraction and final washing. A little xanthophyll and carotin remained in the talc. While shaking, 5 cc. of concentrated methyl alcoholic potash are added to the beautiful, alcoholic chlorophyll solution for the purpose of causing allomerization only, but no saponification; the solution is then permitted to stand. After a lapse of 16 hours the phase test gives only an indistinct, olive green color and after 2–3 days the chlorophyll has been quantitatively allomerized; the change takes place much more quickly in absolute alcohol.

The pigment is now transferred from the alcohol to ether by pouring it in in portions and then carefully diluting with water. After the alcohol has been washed out the volume of the ether amounts to 3–4 l. After drying with sodium sulphate the ethereal solution is vigorously shaken in a large powder-flask with 100 cc. of concentrated methyl alcoholic lye. The chlorophyllin salt separates, after some time, upon the walls and bottom as a viscous liquid. When the ethereal solution is quite clear and a test sample, on shaking with water, turns pure yellow without any fluorescence, the mother liquor, which forms a good extraction material for carotin and xanthophyll, is decanted. The green mass is washed twice with ether, while rotating the flask, and shaken with 1 l. of absolute alcohol. As a result of this treatment the allomerized chlorophyll becomes fine grained and crystalline so that it can be easily filtered from the alcohol, which is relatively poor in pigments, and subsequently washed with absolute alcohol.

The yield of desiccator-dried potassium chlorophyllin amounts to 33–37 g., which is 29.8–33.4 g. when high vacuum dried; *i.e.*, 6 to 6.7 g. from 1 kg. of dry leaves. The salt is 88 per cent pure; *i.e.*, it contains 12 per cent of colorless, admixed material. The splendid, steel-blue lustered preparation dissolves in water with a brilliant green color without any fluorescence. It is free from yellow pigments; when acidi-

fied it yields weakly basic cleavage products only, whose ethereal solution does not color 6 per cent hydrochloric acid.

Crude Potassium Chlorophyllin a.

Potassium chlorophyllin is obtained directly by the saponification of a not too dilute, alcoholic solution of non-allomerized chlorophyll which has been extracted from the crude chlorophyll-talc mixture. Under these conditions the hydrolysis of component *b* leads largely to the iso-compound, which is more soluble and remains in the mother liquor. In this way, preparations of potassium chlorophyllin are obtained that are about 9/10ths *a* component. Chlorophyllin *b* may still be detected therein upon fractionation of the cleavage products with 17 per cent hydrochloric acid. Such a chlorophyllin preparation is of value for obtaining rhodophyllin.

The crude chlorophyll that has been precipitated upon talc is brought into solution by shaking it in a flask with no more absolute alcohol than necessary; this solution is then saponified with considerable concentrated methyl alcoholic lye; for example, with 50 cc. for the pigment from 1 kg. of leaf meal. The chlorophyllin salt begins quickly to precipitate; on standing a half day it forms a bluish black, granular, crystalline powder; it is subsequently washed with absolute alcohol.

The yield of chlorophyllin salt (per kg. of leaf meal) amounts to 4–5 g. It contains 85–90 per cent of the pure potassium compound.

Pure Potassium Chlorophyllin a.[7] The salt was obtained in a crystalline condition from petroleum-ether solutions of chlorophyll *a*, which had been obtained by fractionation of the mixture of chlorophyll components. The petroleum-ether solution of 3 g. of the substance was thoroughly shaken with 10 cc. of 7 per cent methyl alcoholic potash. The chlorophyll went into solution in the lye with a brown color, the chlorophyll green color then returned in a few minutes, and the potassium compound crystallized abundantly in beautiful, dark blue lustered plates, which under the microscope showed a pure green color by transmitted light. The petroleum ether was decanted and the salt washed out with the least possible amount of methyl alcohol, subsequently washing upon the filter with absolute alcohol.

The methyl alcoholic mother liquor was diluted with a little methyl alcohol and saturated with carbonic acid. In this way almost all the

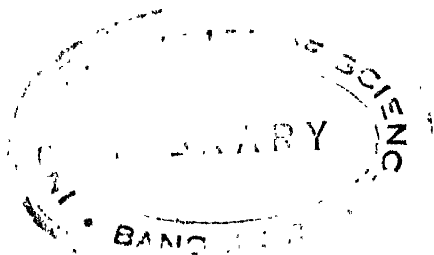
[7] Ann. d. Chem. 382: 157. 1911.

remainder of the potassium salt separated together with potassium methyl carbonate, and it was finally obtained in a rather pure state by fractional crystallization from methyl alcohol.

The first crystallization of potassium salt was free from potassium carbonate. The beautiful preparation was reprecipitated by taking it up at ordinary temperature with 100 cc. of absolute methyl alcohol (in which process a little colorless material remains), strongly concentrating in a vacuum and precipitating with absolute alcohol. The yield then amounted to 0.8 g.

The chlorophyllin salt is very easily soluble in water with a splendid blue green color; rather easily in cold methyl alcohol, easily in warm; difficultly in cold ethyl alcohol, only a little more easily in hot, and with very great difficulty in pyridine. The preparation is not wholly free from methoxyl and therefore corresponds only approximately to the formula, $(C_{31}H_{31}N_4Mg)(COOK)_3$ or $(C_{31}H_{29}N_4Mg)(COK)(CO_2K)_2$.

On careful acidification with a primary phosphate chlorophyllin is set free; on decomposition with hydrochloric acid, the unstable, olive green phytochlorin *g* is formed. This is not extracted abundantly from ether by hydrochloric acid until the latter's concentration is 11 per cent. This solution in hydrochloric acid has a bluish tinged green color.



XIX. INTRODUCTION OF MAGNESIUM INTO THE DERIVATIVES OF CHLOROPHYLL.[1]

Metals may be easily introduced by substitution into the chlorophyll derivatives that have been formed by the elimination of magnesium, for example, into pheophorbide, phytochlorin, phytorhodin and the various porphyrins. Complex compounds are thus formed which are distinguished by their stability in acid and alkaline media. Such metallic compounds, for example, result from the action of copper, zinc and iron salts in glacial acetic acid (often in alcoholic solution also) upon the nitrogenous carboxylic acids and their esters. Important changes in color and in basic properties go hand in hand with the formation of these complex compounds. The fact that metals are taken up is shown by the fact that the slightest traces of certain metals, such as zinc or copper, can be detected by the aid of phytochlorin *e*. It is only by means of special care that the magnesium-free chlorophyll derivatives may be obtained wholly free from ash.[2] The zinc content of glass is troublesome and the copper content of the solvents is disclosed.

For example, in order to detect copper in the pure methyl alcohol of commerce phytochlorin *e* is dissolved in it and permitted to stand for some time. The reaction takes place more quickly in the presence of pyridine. The chlorophyll derivative is then again transferred to ether and the excess phytochlorin separated quantitatively by washing with 10 per cent hydrochloric acid. The intense blue green, copper compound of phytochlorin *e*, which is stable towards acids, remains in the ether.

The fact that glass which contains zinc gives up this metal very easily to alkalies[3] is used for its detection in them; for example, even dissolving pure potassium hydroxide in methyl alcohol (50 g. KOH in

[1] Paper XXI.

[2] Spatulas that are made of base metals must not be used in working with the chlorophyll derivatives. In most cases we used spatulas of refined silver and, for special purposes, thin wooden-handled spatulas of an elastic alloy of gold-platinum (90 per cent gold), which were manufactured for us by W. C. Heraeus.

[3] For the first of such observations, see Ann. d. Chem. 358: 249. 1908.

100 cc.) in the vessels to be tested is sufficient. If, then, 5 mg. of pheophytin are saponified in a silver, or pure potash glass test-tube with about 5 cc. of this hot lye, or if 3–4 mg. of phytochlorin *e* are dissolved therein, the solution remains green in the presence of zinc, even on dilution with water, and on acidification with monosodium phosphate, ether extracts the pigment with a pure blue color and an intense, dark red fluorescence. Excess chlorine *e* can be removed from this by means of 5 per cent hydrochloric acid. The zinc compound is slowly decomposed when its ethereal solution is shaken with 12 per cent hydrochloric acid; quickly with 20 per cent hydrochloric acid. Besides this characteristic property, the spectrum of the complex compound also serves for its identification; the spectrum is similar to that of chlorophyll and wholly different from that of the magnesium-free derivatives. The principal bands are at $\mu\mu$ 653–623, 603 . . . 589, 560 . 542 and 523 . . 507. Many glasses used in the construction of laboratory apparatus and flasks are by means of this test found to contain zinc.

The complex compounds of many metals are unstable toward acids and require other conditions for their formation. Phytochlorin in methyl alcoholic solution produces, with an excess of anhydrous barium hydroxide, the intense blue green solution of a barium compound and even alkalies are capable of forming analogous compounds. These are formed when phytochlorin and phytorhodin are dissolved in very concentrated alcoholic potash. They are again decomposed, however, by mere dilution with alcohol. All degrees of stability, therefore, are realized in the complex metallic compounds of this group. The magnesium complex of the natural pigment lies between the extremes of the exceedingly unstable potassium compound and the copper compound of unprecedented stability.

Willstätter and Forsén have recently succeeded in introducing magnesium into the metal-free chlorophyll derivatives and have thus succeeded in accomplishing a small step in the synthesis of chlorophyll. A useful reagent for this purpose is magnesium oxide in the presence of very concentrated alkali. Magnesium dissolves in molten potassium hydroxide with the evolution of hydrogen to form a clear mass (for example, 0.1 g. of the metal in 25 g. of KOH); when more metal is dissolved the melt becomes opaque. Magnesium oxide, also, is soluble, though only in traces, in methyl alcoholic potash, but it is quite soluble in molten caustic potash (for example, 0.16 g. MgO in 25 g. of KOH).

The melt may perhaps contain a compound of magnesium with the caustic potash, which can be designated a "magnesiate": $Mg(OK)_2$

If pheophytin or any other metal-free chlorophyll derivative is heated in a silver test-tube with methyl alcoholic potash and magnesium oxide, a series of the various phyllins is formed which, on careful acidification, dissolve in ether with green, bright blue and red colors and intense fluorescence. In this way there was obtained on a large scale from phytochlorin *e*, first, the corresponding chlorophyllin and, then, three different phyllins, dependent upon whether the reaction temperature was 180, 200 or 220°; namely, the dicarboxylic acids, cyanophyllin and erythrophyllin and, finally, the monocarboxylic acid, phyllophyllin. Analogously, in the *b* series rubiphyllin was formed from phytorhodin *g* and pyrrophyllin from phytorhodin *k*.

The method can also be successfully used for the introduction of magnesium into the porphyrins that are obtained from hemin and, furthermore, it can be extended to the formation of other complex metallic compounds; for example, to the introduction of iron into the porphyrins by heating them with methyl alcoholic potash and iron oxide.

The method has one disadvantage. The use of strong alkalies and high temperatures excludes the formation from the magnesium-free derivatives of chlorophyll itself as well as that of its first transformation products. A second method for the introduction of magnesium is successful here; it depends upon the reaction of chlorophyll derivatives with Grignard's reagents.

As is known, amino and imino groups can be quantitatively substituted by the aid of a magnesium alkyl-halide. For example, methyl magnesium iodide reacts with the pure *a* component of pheophytin, forming insoluble magnesium iodide compounds. A precipitate that contains 2 atoms of magnesium is formed by the action of 1 mol. of $MgCH_3I$; when double the quantity of the Grignard reagent is used, a salt with 4 atoms of magnesium is obtained. Pheophytin alone was again formed in all cases from the two precipitates that were thus obtained, regardless of whether decomposition was brought about by water, acids, salt solutions or other reagents.

The result obtained by employing a still greater quantity of magnesium methyl iodide (for example 8 mol.) was entirely different. In this case the pheophytin is precipitated quantitatively in the form of a compound which, when decomposed with water and ether, produces a

chlorophyll green; *i.e.*, a magnesium-containing solution. If the same experiment is carried out with chlorophyll itself a precipitate that is rich in magnesium and iodine is similarly formed which, on decomposition, produces a chlorophyll preparation that is unchanged as regards its magnesium-content and its phytol number as well as its methoxyl number. The various ester groups, therefore, remain intact for the moment in the treatment with magnesium methyl iodide.

Nevertheless, the result could not yet be used because the magnesium compounds that were thus formed were not wholly undamaged chlorophyll, for they did not give the "brown phase" of chlorophyll when treated with potash; they had, in fact, been allomerized by the action of the basic magnesium compounds that were formed in the hydrolysis. It was only when Willstätter and Forsén undertook the rapid decomposition of the precipitate, that had been obtained by means of the Grignard solution, with an excess of monosodium phosphate that the pheophytin component *a* was successfully converted into the pure chlorophyll component *a*.

All the possible porphyrins can be transformed in the same manner into the corresponding phyllins.

Pheophytin *b* undergoes a remarkable transformation when acted upon by Grignard's reagent; namely, a change to the series of the *a* components before the magnesium complex is formed; this is not the result of a simple reduction to the oxygen-poorer component itself but is probably due to the addition of the magnesium alkyl-halide to a carbonyl group.

1. Introduction of Magnesium by Means of Magnesium Oxide and Caustic Potash.

Formation of Cyanophyllin. Pure potassium cyanophyllin was obtained from phytochlorin *e* by heating it for 4 hours at 180° with methyl alcoholic potash and magnesium oxide. The violet modification of the chlorin (lactam hydrate) was used and heated in quantities of 1 g. with the same weight of magnesia and 10 cc. of concentrated methyl alcoholic potash in a silver container in an autoclave. The potassium salt of the magnesium compound that was formed was then completely precipitated by the addition of 50 cc. of water and a little sodium chloride solution. The crude potassium cyanophyllin, with which magnesium was still mixed, was taken up with 1 l. of water and separated by shaking with 3 l. of ether in a separatory funnel, in

order that it might be carefully acidified with primary sodium phosphate. When liberated, the phyllin was dissolved by the ether with a pure blue color and very strong, bright red fluorescence. The solution was washed with much water, dried with sodium sulphate, and concentrated to 10 cc. It was then necessary to filter it again since cyanophyllin decomposes similarly to phyllophyllin, in fact, even more easily than phyllophyllin since it changes into magnesium salt, composed chiefly of the corresponding porphyrin. The ethereal solution gradually becomes turbid and gray, even on standing in the cold. In this way the yield of free phyllin is lessened. Only 0.4 g. was obtained, although the potassium salt was formed almost quantitatively.

The concentrated, ethereal, cyanophyllin solution gave, with petroleum ether, a greenish blue, flocculent precipitate, whose analysis gave the atomic ratio N_4: 0.95 Mg.

Phyllophyllin from phytochlorin e. If on heating with magnesium oxide and methyl alcoholic potash, the temperature is kept for 6 hours at 220°, there is formed, by the splitting off of carbon dioxide, the easily decomposable monocarboxylic acid, which had heretofore been analyzed only in the form of its salt. After 1 g. of phytochlorin was heated with 15 cc. of lye and 1 g. of magnesium oxide, the potassium salt that was formed was precipitated completely with 50 cc. of water. It was brought into solution by triturating with water and ether, and carefully acidified with phosphate. The ethereal solution contained an admixture of the dicarboxylic phyllin which could be removed by shaking it 4 times with considerable 0.025 per cent ammonia. The ether was then again thoroughly shaken with primary phosphate, washed, and dried. The ethereal solution was concentrated to 15 cc. and again filtered, after which 0.5 g. of pure phyllophyllin was successfully precipitated by the addition of 400 cc. of petroleum ether. It was identified by analysis and by the characteristic solubility relations of the calcium salt.

2. Introduction of Magnesium by Means of Grignard's Compounds.

Reaction of pheophytin with methyl magnesium iodide. The Grignard reagent used in the following experiments required 11.94 cc. of 0.1 *N* H_2SO_4, corresponding to 0.198 g. of $MgCH_3I$, for the neutralization of one cubic centimeter; that is, 1 mole for each gram of pheophytin *a*.

This quantity of magnesium solution gave, with the ethereal solution (700 cc.) of 1 g. of pheophytin component *a*, an incomplete precipitation of dark floccules which formed, after isolation in the apparatus of E. Beckmann and Th. Paul[4] under conditions where moisture was completely excluded and after drying, a bluish black powder (0.6 g.), which was insoluble in all solvents. Considerable pheophytin, therefore, remained in the mother liquor.

The desiccator-dried preparation contained 4.46 per cent N, 3.85 per cent Mg and 18 per cent I, corresponding to the atomic ratio



$$N_4 : 2.0\ Mg : 1.8\ I.$$

Chlorophyll could not be obtained by the decomposition of this magnesium compound by any possible reagent because pheophytin alone was reformed from it.

Even double the quantity of methylmagnesium iodide (2 cc. for 1 g. of pheophytin *a* in 700 cc. of ether) was not sufficient for complete precipitation of the pheophytin; it gave a precipitate that was much richer in magnesium. This precipitate, also, was insoluble in the ordinary solvents, and water or dilute phosphoric acid likewise regenerated chiefly pheophytin from it.

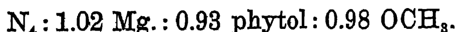
This second preparation contained 3.92 or 3.97 per cent N, 6.88 per cent Mg, 31.4 per cent I and gave the ratio



$$N_4 : 4.0\ Mg : 3.5\ I.$$

When the quantity of Grignard reagent was again doubled; *i.e.*, 4 moles of methylmagnesium iodide were allowed to act, the precipitation of pheophytin was quite complete and the precipitate showed an entirely different behavior towards water and acids; one atom of magnesium remained in the molecule when it was hydrolyzed.

In connection with the preliminary work on this method for the introduction of the metal, mention should also be made of the fact that pure chlorophyll can be obtained again in an undamaged state after the pheophytin has been precipitated with an excess of methylmagnesium iodide (about 8 moles) and the precipitate has been decomposed with monosodiumphosphate. No change occurred in the magnesium or in the phytol content or in the $COOCH_3$ group because the analysis of the chlorophyll obtained in this way gave the ratio



$$N_4 : 1.02\ Mg. : 0.93\ phytol : 0.98\ OCH_3.$$

[4] Ann. d. Chem. 266: 1. 1891.

Transformation of Pheophytin a into Chlorophyll a.

The initial material for this experiment was a preparation of the pheophytin component *a*, which easily produced phytochlorin *e* on hydrolysis. The ethereal solution of this pheophytin (1 g. in 1 l.) was mixed with the Grignard reagent, drop by drop, till a sample in a test-tube, on shaking with dilute phosphoric acid, no longer produced an olive tinged, but a pure blue, solution. All the pheophytin was then also precipitated as clear green floccules. Decomposition of the intermediate product, which contains much iodine and magnesium, gives a beautiful result if it is done quickly with a large excess of primary phosphate; for example, by adding 1 l. of 10 per cent monosodium phosphate solution, all at one time, and immediately agitating vigorously. The ethereal solution now shows the beautiful blue color of the chlorophyll component *a*. After this was thoroughly washed with considerable water and then dried, the chlorophyll that had been formed (0.8 g.) was precipitated by means of petroleum ether.

Upon direct comparison it agreed exactly with the pure chlorophyll *a* component; namely, in composition, in the color phenomena of the solution, in the phase test and in the cleavage test.

The Methyl Ester of Phyllophyllin from the Porphyrin Ester.

The formation of this ester may be adduced as an example of the introduction of magnesium into the complex compound by the aid of Grignard's solution, a procedure which is applicable to all the porphyrins.

The methyl ester of phylloporphyrin (0.7 g. in 1 l.) was dissolved in boiling, absolute ether and, after cooling, precipitated, drop by drop, with methylmagnesium iodide till a small test sample, after careful decomposition with primary phosphate, no longer reformed even a trace of the phylloporphyrin ester; *i.e.*, nothing could be extracted any more from an ethereal solution by 2 per cent hydrochloric acid.

The substance was precipitated quantitatively as raspberry red floccules; it was not necessary to isolate these, but the suspension was simply shaken in ether with an excess of monosodium phosphate and the bluish tinged, red fluorescent solution was then concentrated considerably after the usual washing and drying. It was only after all the solvent except a few cubic centimeters had been removed by dis-

tillation that the methyl ester of the phyllophyllin crystallized in rhombic-shaped plates, the obtuse angles of which were often rounded. When once crystallized, the substance was only very slightly soluble in ether; it could be recrystallized beautifully from alcohol.

Action of Grignard's reagent upon the compounds of the b series.[5] Pheophytin *b* is, analogously to the *a* component, completely precipitated by 4 moles of Grignard reagent ($MgCH_3I$) as an addition product in the form of brownish red floccules; these, however, unlike the case of the *a* precipitate, do not turn green, even when boiled with double the quantity of magnesium solution; that is, they are not converted into the complex, magnesium compound. On shaking the suspension with a monosodium phosphate solution, the ether becomes colored with the brownish red color of the unaltered pheophytin *b*.

When a large excess of methylmagnesium iodide (0.4 g. $MgCH_3I$ in 25 cc. of ether) acts upon an ethereal solution of pheophytin *b* (0.025 g. in 25 cc.), the product of the reaction precipitates immediately in brown floccules which very quickly turn yellow. When immediately acidified, an olive brown, ethereal solution is formed, which reminds one very much of pheophytin *a*, and hot saponification with methyl alcoholic potash of a sample that has been previously evaporated gives a four-fifths yield of a cleavage product which looks like chlorin *e*. In ether it is olive green and, in 3 per cent hydrochloric acid, blue, with a greenish tinge.

But a direct comparison with pure phytochlorin *e* permits distinct differences to be recognized. The cleavage product that is obtained from pheophytin by means of methylmagnesium iodide is somewhat more strongly basic (HCl number 2–2½); it is more blue in hydrochloric acid and it has a slightly more yellowish tinge in ethereal solution.

Only after the precipitated product of the reaction with Grignard's solution stands for a long period of time (though more quickly if warmed) do the initially yellow floccules acquire an olive, and finally bluish green, color as well as dissolve in ether with a beautiful bluish green color when carefully acidified. This solution agrees with chlorophyll *a* in color and phase but the cleavage test yields, in addition to rhodin *g*, the same cleavage product that differs slightly from true phytochlorin *e* as is obtained before the introduction of magnesium.

[5] Unpublished.

Chlorophyll *b* is also precipitated by Grignard's solution and transformed, when a great excess of the magnesium solution is used, into a derivative of the *a* series which, on systematic decomposition, leads to the same cleavage product as pheophytin.

The transformation in the case of phytorhodin *g* proceeds without the production of such secondary products as are formed in the cases of chlorophyll and pheophytin by saponification of the ester groups or by allomerization. Because it is difficultly soluble in ether the phytorhodin *g* is dissolved in dry pyridine and it is treated with considerable Grignard's reagent, since pyridine also reacts with the reagent. 0.02 g. of rhodin *g*, dissolved in 1 cc. of pyridine, were added to 100 cc. of a 1.5 per cent methylmagnesium iodide solution. The rhodin was precipitated as green floccules together with the addition product of the pyridine and, when acidified, gave a full yield of chlorin with a basicity of 2–2.5.

The reactive, oxygen-containing group is, consequently, as much a constituent of the chlorophyll itself as of the phytorhodin *g* that has been formed by relactamerization. It is the group which the corresponding compounds of the *a*-series lack because true phytochlorin *e* is formed from them even when they are acted upon by a large excess of Grignard's solution.

Homologous Grignard compounds lead to various end products; the basicity and color of the product of the reaction changes with the alkyl of the Grignard compound that attaches itself to a carbonyl.

Under the conditions described, magnesiumbrombenzene, for example, gives an ether-soluble, addition product with phytorhodin *g* and, on acidification, a pigment that is yellowish olive in ether and which does not dissolve abundantly in hydrochloric acid below a concentration of 5–6 per cent, dissolving in this with a bluish-green color.

Magnesium hydride (probably containing MgHI) prepared by the method of P. Jolibois[6] produces with phytorhodin *g* in pyridine, when boiled and acidified, a very strongly basic product, two-thirds of which is extracted from ether (in which it dissolves with great difficulty with a yellowish olive color) by even 0.5 per cent hydrochloric acid.

[6] Chemisches Zentralblatt review 155: 213. 1912 and 155: 353. 1912.

XX. SYSTEMATIC DECOMPOSITION OF CHLOROPHYLL BY MEANS OF ALKALIES: PHYLLINS AND PORPHYRINS.

1. Summary.

The magnesium complex of chlorophyll remains intact when it is heated with alkalies. The phyllins, a series of magnesium-containing carboxylic acids that have beautiful blue and red colors, are obtained in this way. The complexly bound magnesium of the phyllins is very reactive toward acids. An analogous, second series of acid compounds, which, on account of a certain similarity to the iron-free derivatives of hemin, have been designated as porphyrins, is formed from the phyllins when the magnesium is eliminated, a process that is accompanied by a considerable change of color.

Willstätter and Pfannenstiel[1] and Fritzsche[2] investigated the systematic decomposition of chlorophyll when heated with methyl alcoholic potash and found the end products to be two isomeric monocarboxylic acids, having the formula $(MgN_4C_{31}H_{33})(COOH)$, phyllophyllin[3] and pyrrophyllin. These cannot be converted into one another but, as reported in Chapter XXIII, produce, by splitting off carbon dioxide, the two parent substances of the group:

Etiophyllin $MgN_4C_{31}H_{34}$,
Etioporphyrin $N_4C_{31}H_{36}$.

Pyrrophyllin is not formed from the one chlorophyll component and phyllophyllin from the other but both are formed from either chlorophyll component, depending upon the conditions of decomposition.

[1] Paper V.

[2] Paper VIII.

[3] We were obliged to use this unusual name because this phyllin corresponded to the previously known phylloporphyrin. The names, glauco- and cyanophyllin, derived from the Greek expressions for blue and the prefixes of the names, rhodo-, erythro-, rubi-, pyrrophyllin, are derived from various terms for red.

The lactam theory of the brown phase (see Chapter I) offers an explanation for this; it assumes that in the saponification, for example, of the chlorophyll component *a* to chlorophyllin the lactam group $\begin{matrix} CO\gamma \\ | \\ NH\delta \end{matrix}$ closes and the α and β carboxyls are released while in its saponification to isochlorophyllin, the lactam group $\begin{matrix} CO\alpha \\ | \\ NH\gamma \end{matrix}$ closes and the γ and β carboxyls are released.

These lactam groups disappear in the course of the decomposition to the blue intermediate products and, furthermore, the β carboxyl is lost.

The difference between the monocarboxylic phyllins, therefore, is probably due to the variation above stated; *i.e.*, to the different location of the α and γ carbozyls.

The two isomers, phyllo- and pyrroporphyrin, do not agree in composition alone. In addition to some characteristic differences that are grouped in the following table, which makes a clear description possible, so many characteristics in common are shown that it was difficult to decide on the one that corresponds to the phylloporphyrin of the literature.

	Pyrroporphyrin	Phylloporphyrin
Hydrochloric acid number	1.5	0.5–0.75
Solubility of the hydrochloride in HCl of moderate dilution	difficultly soluble	easily soluble
Color of a concentrated solution in dilute HCl	bluish red, tinged blue	brownish tinted red, greenish tinged
Dyes silk	copper red	dichroic, greenish, bronze brown and coppery
In glacial acetic acid (cold)	rather difficultly soluble	very easily soluble

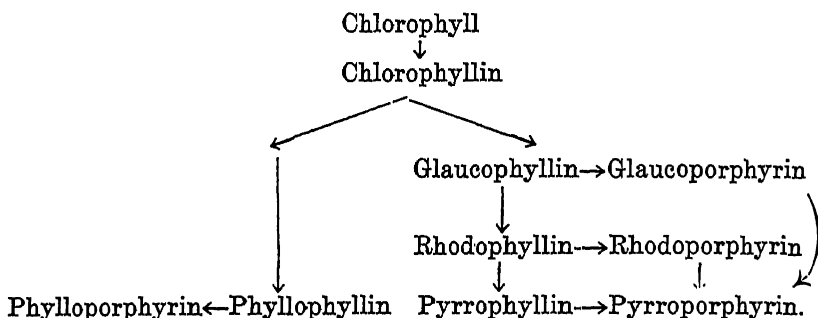
In an investigation of the action of alkalies upon chlorophyllan at high temperatures F. Hoppe-Seyler[4] obtained a compound that was very similar optically to his hematoporphyrin. He introduced the name, phylloporphyrin. E. Schunck,[5] ten years afterwards, and later,

[4] Zeitschr. f. physiol. Chem. 4: 193. 1880.
[5] Proc. Roy. Soc. 50: 302. 1891.

jointly with L. Marchlewski,[6] prepared a purer decomposition product by heating chlorophyll derivatives with alkali and transferred the designation of Hoppe-Seyler to it. Our pure phylloporphyrin partly agrees with the description given for it in the literature because, according to the statements of Schunck and Marchlewski, porphyrin is very easily soluble in mineral acids and in glacial acetic acid and "ether does not extract anything from these solutions when it is shaken with them, not even when the solution is greatly diluted with water." On the other hand, the statements regarding the absorption spectrum fit pyrroporphyrin better, even though the observations are not sufficient for certain identification since they refer to a single unknown concentration and thickness only.

It is very probable that the more strongly basic of our two porphyrins is identical with the phylloporphyrin of the literature and that the earlier authors did not have any pure porphyrin in their possession. Still, the only good method for producing the pure isomeric monocarboxylic acids on a preparative scale depends upon the separation of the corresponding phyllins, which are distinguished characteristically by their solubility and the separation of their calcium and ammonium salts.

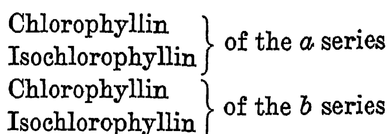
In the paper by Willstätter and Fritzsche the following synopsis for the decomposition of chlorophyll by alkalies has been given:



We have, together with M. Utzinger, L. Forsén and M. Fischer, repeatedly checked the investigation of Willstätter and Fritzsche and corroborated their statements; the gaps in the above systematic arrangement have been filled as a result of unpublished experiments that are briefly described in what follows.

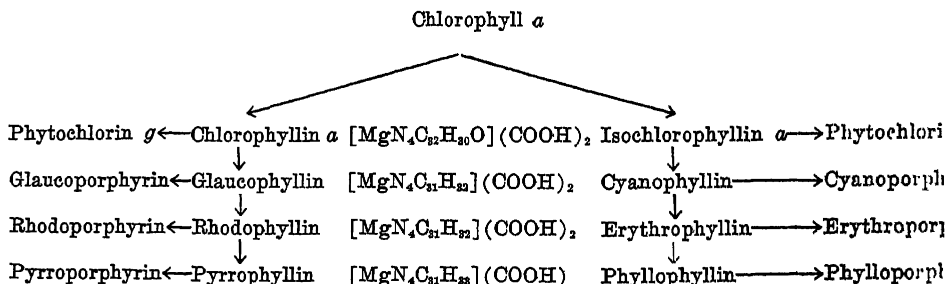
[6] Ann. d. Chem. 284: 81. 1894 and Proc. Roy. Soc. 57: 314. 1895.

It was only after the pure *a* and *b* chlorophyllides had been isolated that it became possible to use the four different chlorophyllins; namely,



(of which the third only has not been prepared in the pure state since as produced it was accompanied by the first or the fourth), as initial material for the action of alkalies and to establish the more important steps of their systematic decomposition.

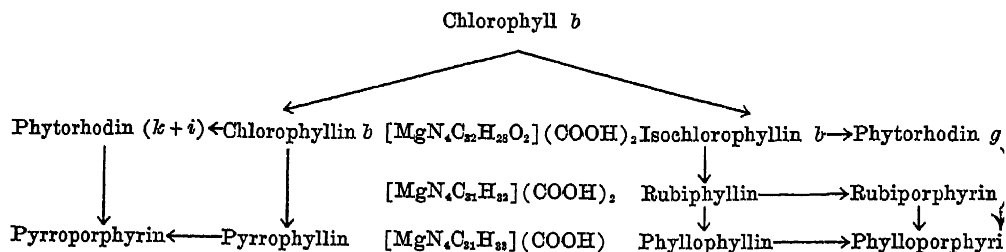
The following table gives, without gaps, for the chlorophyll *a* series a summary of the systematic decomposition which leads to the di- and mono-carboxylic acids.



The systematic decomposition of the *b* chlorophyllin components to the phyllins was much more difficult to accomplish since, in the compounds of the *b* series, an additional oxygen-containing group is present and must be reduced without the elimination of carbonic acid. This was successfully accomplished by heating with concentrated, methyl alcoholic potash and the addition of pyridine; phytorhodin *g*, also, which is unstable toward alkalies, can be decomposed to phylloporphyrin when pyridine is present.

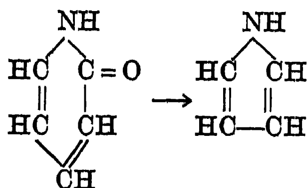
There was prepared in the *b* isochlorophyllin series and preceding the final product, phyllophyllin, pure rubiphyllin, an intermediate product that corresponds to erythrophyllin. This was compared, as to characteristics, with its isomers. Pyrrophyllin was obtained analogously from *b* chlorophyllin, though not in a pure state but accompanied by phyllophyllin because potassium isochlorophyllin *b* was also present in the initial material.

The introduction of magnesium by Willstätter and Forsén's method has made some of the phyllins more easily obtainable from the pheophorbides, phytochlorins and phytorhodins.



All the phyllins and porphyrins that are formed from the chlorophyllins at an elevated temperature are defined as carboxylic acids of the parent substances, etiophyllin and etioporphyrin. But in regard to their formation an important question must still be solved. Chlorophyllin *a*, for example, in which the relations are somewhat simpler than in *b*, loses very probably, when decomposed to glaucophyllin and rhodophyllin, a carbon atom that is bound with oxygen. The question is, is it simply a carbonic acid cleavage in the later steps of the systematic decomposition, as in the formation of pyrrophyllin from rhodophyllin or of etiophyllin from pyrrophyllin? This is improbable because the phytochlorins and phytorhodins are decidedly different from all the porphyrins, which show a great resemblance to each other. There is rather to be assumed a fundamental change of the molecule when the chlorophyllins are altered to form the blue compounds.

The lactam group of phytochlorin *e* can be formed either by a carboxyl which is a substitute in a nitrogen-containing ring, or by a carboxyl which is itself a constituent of such a ring. The significant difference between phytochlorin and the porphyrins can only be explained by the second assumption. When the chlorophyllins are decomposed a carbonyl group appears to be eliminated and a new pyrrol ring formed somewhat after the following scheme, which shows only the essential part of the process:



It is still uncertain whether glaucophyllin and rhodophyllin have exactly the same composition; the former, though it may be formed by the elimination of carbon monoxide alone, may also have two hydrogen atoms less.

2. Preparation of the End Products,[7] Pyrro- and Phyllophyllin, from Chlorophyll (a + b).

The separation of the monobasic from the dibasic phyllins is made possible by the following very characteristic differences:

Pyrro- and phyllophyllin give potassium salts that are soluble in alcohol; potassium rhodophyllin and potassium glaucophyllin are insoluble in alcohol.

Pyrro- and phyllophyllin are not extracted from ether by dilute ammonia.

The monobasic phyllins can be separated by means of the difference in the solubilities of their ammonium salts in ether, but particularly on account of the solubility of calcium phyllophyllin in ether. Besides, they are not formed together under the experimental conditions given.

The alkali salts of the compounds that have only one carboxyl are easily split hydrolytically by water.

Pyrrophyllin from Chlorophyllin (a + b).

20 g. of crude chlorophyllin salt, containing 16 g. of the pure potassium compound, are dissolved in 400 cc. of 35 per cent methyl alcoholic potash (made from KOH that has been purified with alcohol) by rotating and boiling in a silver beaker and slowly heated in an autoclave to 130° C. The temperature is now kept for two hours between 130° and 135° and, in the course of another hour, increased to 170° C. It is then held for two hours at 170–180°, increased, in the course of 1½ hours, to 195°, and held for another hour below 200° C. These temperatures are determined in a silver thermometer shield, which projects into the heated mass. There is a distinct difference if the temperature is determined within the reacting liquor or in the oil bath of the autoclave. The manner of heating is decidedly not a matter of indifference as sufficient time must elapse during the individual steps of the decomposition; in particular, the experiment is successful only when the mass is held for a considerable lapse of time at a temperature of about 130°. The dicarboxylic phyllin appears to be formed

[7] Unpublished method.

by a peculiar transformation of the molecule and not by simple cleavage of carbonic acid.

After cooling, the product of the reaction gives only a very faint odor of hemopyrrol. The end of the decomposition is tested for by means of 0.03 per cent ammonia after the test sample has been transferred into ether. This is colored but slightly when only a little dicarboxylic acid remains. In experiments that have turned out well the ethereal solution is a beautiful violet red, with a bright yellowish red fluorescence; after decomposition of the phyllin with acid, 2 per cent hydrochloric acid extracts 80–85 per cent of the corresponding porphyrin from the ether.

The half-congealed contents of the autoclave are diluted slowly by stirring them with double their volume of water and the potassium salt of the pigment is precipitated almost entirely as violet, crystalline floccules as a result. This is filtered upon the suction filter and then washed with a little water. The salt is stirred with 100–200 cc. of water with the addition of some ether, forming a uniform, thin paste; this is diluted with 0.5 l. of alcohol and the fine suspension introduced into 5 l. of ether. This is then mixed with 20 g. of monosodium phosphate crystals and shaken. A large portion of the pyrrophyllin passes into the ether. The bottom layer is diluted with more water, run off into a second separatory funnel and shaken with ether and some more phosphate. By this procedure the formation of a flocculent precipitate is avoided. The ethereal solution is washed with moderately dilute monosodium phosphate and with water and the secondary product, rhodophyllin, is then extracted with 0.03 per cent ammonia. The first extracts are a beautiful cherry red; subsequent extracts do not color the ammonia. It pays to work up the ammoniacal solutions for rhodophyllin; they are mixed with a little alcohol, acidified with phosphoric acid and extracted with ether. On concentration to 15 cc. the rhodophyllin separates in violet, lustrous crystals.

The purified pyrrophyllin solution is freed from ammonia by shaking with phosphoric acid and, after washing, it is dried with ignited sodium sulphate. It is then quickly concentrated on a water-bath to 1 l., filtered and evaporated in a vacuum at ordinary temperature to 50 cc., after which it is slowly precipitated with 1 l. of petroleum ether, while stirring. After standing in the ice-box for a day the mother-liquor still contains only a little phyllin; the violet red, pulverulent

precipitate is washed while upon the filter with low boiling petroleum ether.

The yield amounts to 8.5 g. of pure pyrrophyllin (that is, three-fourths of the theoretical) and 0.6 g. of rhodophyllin.

The phyllin was obtained less completely, and not quite so easily, by crystallization instead of by precipitation from the strongly concentrated ethereal solution. It crystallizes from the bright red fluid in very pure aggregates of steel blue lustered, or reddish gray blue, acute angled plates.

Preparation from rhodophyllin. 5 g. of crude crystals of rhodophyllin were heated with 200 cc. of concentrated methyl alcoholic potash for 3½ hours in a silver beaker by means of a Pfungst autoclave to 225–230° C. (oil bath temperature). The deep red, alkaline fluid gives, on dilution with 300 cc. of water, a complete precipitation of flocculent potassium pyrrophyllin.

Recrystallization.[8] When recrystallized from absolute ether, the pyrrophyllin undergoes a change which is not recognizable by changes in its chemical behavior but by changes in its solubility and color. When pyrrophyllin is treated with solvents the color becomes less and less bluish in tinge and the absorption in the red portion of the spectrum diminishes. The crude solution of the material is bluer than that of rhodophyllin, but as soon as the crude product is redissolved this solution of pyrrophyllin is less blue; it then has a beautiful violet tinge. Recrystallized pyrrophyllin gives a fuchsin-like, pure red, ethereal solution with a yellowish-red fluorescence. This does not have the strong blue tone. This change goes hand in hand with a decrease in solubility in dry ether. The finely powdered, crude product still dissolves very easily and quickly in this; it is also necessary to prepare and filter the solution quickly so that reseparation does not take place too quickly. 3 g. of the substance were dissolved in the course of a few minutes in 150 cc. of absolute ether and concentrated to half this volume. The pyrrophyllin (1.7 g.) then crystallized quickly and, with somewhat further concentration, 0.35 g. more crystallized in very beautiful, red violet, strongly lustrous prisms, which showed a hexagonal outline under the microscope and appeared ruby red by transmitted light. If, on the other hand, moist ether is employed for recrystallization, the pyrrophyllin is as easily soluble as the crude product and it also behaves similarly to the crude product when it is dried.

[8] Ann. d. Chem. 371: 74. 1909.

The preparation that has been recrystallized from anhydrous ether contains ether of crystallization, which it loses with extraordinary difficulty at 105° in a vacuum.

Phyllophyllin from Isochlorophyllin (a + b).

The alkaline isochlorophyllin solution from 10 g. of methyl chlorophyllide (see Chapter XVIII, section 1) is poured into the silver inset of the Pfungst autoclave, which holds 300 cc., and covered with an asbestos plate. This is slowly heated in an oil bath, provided with a thermometer, to 140° and the temperature kept at the cyanophyllin stage 2–3 hours; that is, at 140–150°; it is then slowly increased to 170° (rubiphyllin formation) and, after another hour, to 180–190° (decomposition of the rubiphyllin, formation of erythrophyllin). The temperature is kept constant here for 2–3 hours and finally allowed to rise another 2–3 hours from 190–205° for the transformation of erythrophyllin (18 atm. pressure corresponding to 195°, the temperature within our larger autoclave).

By dilution with double the volume of water the potassium salt was quantitatively precipitated from the product of the reaction in a granular, crystalline form. The salt, after filtration, is triturated with 200 cc. of water and some ether, then diluted with 0.5 l. of alcohol and introduced into 4–5 l. of ether. The very fine suspension of potassium phyllophyllin, which partly dissolves in ether, is acidified by shaking with 20 g. of coarse crystals of monosodium phosphate; the ethereal solution is washed with moderately strong phosphate solution and the alcohol is separated from it with considerable distilled water.

Some dicarboxylic acid, namely, erythrophyllin, is then separated by extraction with 0.03 per cent ammonia. After renewed washing with primary phosphate and water the phyllophyllin is shaken vigorously and repeatedly with 1 to 1.5 l. of lime water. Some floccules of damaged phyllin form the first time this is done. The ethereal solution of calcium phyllophyllin, after being dried over calcium chloride, is evaporated, without having been washed, to about 200 cc., whereupon a portion of the calcium salt precipitates as a fine crystalline paste. The remainder crystallizes almost completely from the filtrate on mixing it with an equal volume of alcohol. The mother liquor contains small amounts of another phyllin whose magnesium-free derivative is green in ether and which is not extracted by hydrochloric acid of less than 7 per cent concentration.

The ammoniacal erythrophyllin extracts are acidified with primary phosphate and transferred to ether; when strongly concentrated the by-product is obtained from this in beautiful crystals; for example, 1.1 g.

The yield of calcium phyllophyllin amounts to about 70 per cent of the theoretical yield; namely, 6 g.

Isolation in the form of the salt is advantageous on account of the stability of this during the evaporation of its solution and when stored. The free phyllophyllin loses its magnesium more easily than the pyrro-compound and most other phyllins. The solution of the free acid can exist only when it is diluted and decomposition to a small extent is evident even when a freshly prepared ethereal solution stands for a short time, its absorption spectrum showing more and more the double bands that are characteristic of phylloporphyrin in the orange.

When the ethereal solution stands for a longer period, and particularly when it is evaporated (even when most carefully concentrated in a vacuum at a low temperature), the whole substance is subject to this cleavage; in the stronger concentration of the phyllophyllin solution this acid has an acidifying effect upon the complex of its own molecule, in consequence of which it quickly decomposes. When the phyllophyllin solution is evaporated a crystallization of phylloporphyrin is obtained while the magnesium that splits off neutralizes a portion of the phyllophyllin to form the ether-soluble magnesium salt. This is found in the filtrate from the porphyrin. The magnesium salt does not have the tinge of the free phyllin; if its ethereal solution is acidified the original color is restored.

Phyllophyllin from Pheophytin (a + b).

It was observed in the earlier works[9] that phylloporphyrin is obtained from pheophytin and phytochlorin *e* when these are heated with methyl alcoholic potash at as low a temperature as 140–150°. One of Willstätter and Forsén's methods described in the preceding chapter is used to introduce magnesium so as to obtain phyllophyllin from the two pheophytin components through the action of magnesium and methyl alcoholic potash, just as its formation from phytochlorin alone, has already been given there. The reaction temperature is higher for the compound that contains magnesium than for the one that does not. In order to systematically decompose the *b* compound to the desired

[9] Ann. d. Chem. 371: 98. 1909 and Ann. d. Chem. 382: 183. 1911.

end product, an addition of pyridine which is effective in the transformation of an oxygen-containing group is necessary.

20 g. of finely divided pheophytin was gradually introduced into 200 cc. of boiling, 40 per cent methyl alcoholic potash which had been mixed with 25 cc. of pyridine and 5 g. of magnesium oxide. When the decomposition is ended, the deep green mass, which is olive colored on dilution, contains phytochlorin *e* and rhodin *g* only, with but traces of floccules. An additional 25 cc. of pyridine was then added and the whole was then heated in the silver vessel of the Pfungst autoclave to 140°. The temperature was kept for 2 hours at 140°, 1 hour at 140–160°, 2 hours at 160°, 2 hours at 175–180°, slowly 180–205° and 3 hours at 205°. Gas formed during the reaction, the manometer indicated 22 and, after cooling, 7 atmospheres of pressure. The reaction produced phyllophyllin chiefly in addition to which, however, there was still quite a little erythrophyllin.

In order to remove the pyridine, the precipitated and filtered potassium phyllin was stirred with water to a paste and poured in a thin stream through 2 l. of ether in a separatory funnel; this treatment was repeated. Further isolation was brought about with primary phosphate and ether containing alcohol, and by converting it into the calcium salt as in the previous section. The yield of calcium phyllophyllin amounted to 6.7 g., to which were added 2.2 g. of erythrophyllin separated by means of 0.03 per cent ammonia.

3. Intermediate Products of Decomposition.

Chlorophyllin a Series.

Glaucophyllin.

Willstätter and Fritzsche[10] produced the first intermediate stage of the decarboxylization of chlorophyll *a* from the potassium salt which they had obtained by means of the cold saponification of stinging nettle extracts. The crude chlorophyllin salt (containing 45 per cent of the pure potassium compound) was heated for 6½ hours at 140° in charges of 30 g. with 250 cc. of concentrated methyl alcoholic potash.

The product of the reaction is dissolved for the most part in the cold lye; on a glass rod it appears deep greenish blue, and even in layers of moderate thickness it is colored red. When diluted with 350 cc. of water the crude potash salt precipitates in green floccules and

[10] Ann. d. Chem. 371: 62. 1909.

can be filtered upon a hardened filter. Chlorophyllin-like material remains dissolved in the mother liquor.

The salt, while still moist, is rubbed in a mortar with alcohol to a paste, diluted with 0.5 l. of alcohol and introduced into 3 l. of ether. The fine suspension is acidified with about 30 cc. of concentrated monophosphate solution; the glaucophyllin easily passes over into the alcoholic ether. The alcohol is then washed out with water; the aqueous layer becomes green and, at the same time, a voluminous colorless impurity separates from the ether in consequence of its becoming poorer in alcohol. Good chlorophyllin material produces beautifully blue solutions of crude glaucophyllin, which exhibit a blood red fluorescence.

Further treatment of the glaucophyllin solution consists in the separation first of more weakly acid and then of more strongly acid chlorophyllin-like accompanying materials. For this purpose it is first extracted with very dilute ammonia; in the case above of rhodophyllin 0.03 per cent was used, but here still more dilute ammonia is recommended. Glauco- and rhodophyllin are easily and completely extracted from dilute ethereal solutions by 0.002 *N* ammonia; on the other hand, only in traces by 0.0002 *N* ammonia. The solution is shaken exhaustively each time with 1½ to 2 l. of about 0.004 per cent ammonia, that is, 5–6 times; if pure chlorophyllin salt has been used the ether is no longer green. The ammoniacal solution is blue and exhibits a very strong red fluorescence; on standing it turns a turbid olive color because the salt precipitates in an extremely fine state of subdivision. The glaucophyllin is again transferred from the ammonia to ether (2 l.) by acidification with very little primary phosphate and the addition of some alcohol, and this solution is thoroughly shaken many times with dilute disodium phosphate (0.02–0.05 per cent). It is evident that the reagent removes an admixture, since it turns blue green in color 3 to 4 times; only traces of glaucophyllin, however, are extracted by even 0.1 per cent disodium phosphate.

The ethereal solution is now pure and has a splendid blue color. After drying with sodium sulphate it is concentrated to 25–30 cc.; the glaucophyllin crystallizes from the very concentrated solution only after it has been cooled and allowed to stand for a short time in the form of lustrous, small prisms having a bluish color by reflected light, similar to that of rhodophyllin. The yield amounts to 1.7 g.

Recrystallization. Glaucophyllin can be recrystallized best from anhydrous ether. In this process it is converted into an ether-insolu-

ble modification and the color of its solution turns more violet, as is the case with pyrrophyllin. 2 g. of the finely pulverized material was boiled in absolute ether for 5 minutes; this was done in two portions, each with 750 cc. of ether. A portion (0.45 g.) of the powder became coarsely crystalline and remained undissolved in this treatment; the glaucophyllin separated from the ether, even during concentration upon the water-bath, in a beautiful, pure crystallization (1.2 g.) consisting of regular prisms having the shape of elongated rhombs.

Rhodophyllin.[11]

Although the content of isochlorophyllin and chlorophyllin *b* in the initial material formerly made the preparation of pure rhodophyllin difficult, a very suitable initial material has now been found in the potassium salt that is obtained from a pure chlorophyll solution when it is subjected to cold saponification. This contains hardly any other chlorophyll material except chlorophyllin *a*.

10 g. of 65 per cent chlorophyllin *a* salt are slowly heated with 200 cc. of 35 per cent methyl alcoholic potash in an autoclave to 130° (temperature of the reacting solution). After two hours the temperature is increased to 160° and for two more hours it is held at not above 165°. Even under these mild conditions, a considerable portion of the rhodophyllin is transformed to pyrrophyllin.

All the phyllin salt is precipitated from the alkaline liquor by dilution with double its volume of water in order to avoid, as in the case of the other phyllins, the acidification of the alkaline fluid. The filtered and washed (with water) flocculent precipitate is mixed with water, double its volume of alcohol and some ether and is introduced into 5 l. of ether. The phyllin is extracted by the ether upon acidification with primary phosphate. A beautiful, red solution is formed, which is then washed with phosphate and with distilled water. The rhodophyllin can be separated from pyrrophyllin by means of very dilute ammonia, which does not take up the monocarboxylic compound; but when there are only small admixtures of pyrrophyllin it is sufficient to let the rhodophyllin crystallize from a dilute ethereal solution.

10 l. of 0.03 per cent ammonia were required for the extraction of the phyllin; the rhodophyllin was transferred from this into considerable alcoholic ether (about 5 l.) again by acidification with solid phosphate and the alcohol was separated by a thorough washing with a

[11] Paper V. The method given here is new.

great deal of water. After rapid concentration to 1 l. the solution was filtered and concentrated further. The rhodophyllin separated from the warm solution, even when this was still dilute, in the form of glistening crystals that had a blue to violet color by reflected light.

After the extraction with dilute ammonia, the ether still retained the pyrrophyllin; the solution was washed with phosphate and with water and precipitated with petroleum ether after it was concentrated.

The yield amounted to 2.2 g. of rhodophyllin and 1.0 g. of pyrrophyllin.

Rhodophyllin can be easily recrystallized from moist ether, 3 l. of which are required for the solution of 2 g. when a reflux condenser is used; the material, after concentration, crystallizes in individually developed prisms which have a bluish red color by reflected light.

The Isochlorophyllin a Series.

Cyanophyllin and Erythrophyllin.[12]

In the isochlorophyllin series the different stages of the systematic decomposition were attained more smoothly and at lower temperatures if the action of the methyl alcoholic potash took place in the presence of a significant quantity of pyridine. Thus, pure cyanophyllin salt is formed in 4–5 hours from the product of the hot saponification when heated in the autoclave with three times its quantity of pyridine and ten times its quantity of methyl alcoholic potash at 150–155°. It is easily soluble in alcoholic potash and also in water and can be precipitated with table salt only as a viscous mass. On careful isolation by means of acid phosphate in the presence of ether a beautifully blue, intensively fluorescent, solution is formed. But this is so unstable that, even when it stands, it soon becomes turbid and gray in consequence of the elimination of magnesium from the complex. Cyanophyllin as such, therefore, can not be separated by evaporation but this can be done by precipitation with petroleum ether.

The analogue of rhodophyllin is much more stable than the stage that corresponds to glaucophyllin.

For the preparation of erythrophyllin, chlorophyll *a* was diluted in gram portions with 3 cc. of pyridine, subjected to hot saponification with 10 cc. of methyl alcoholic potash, and heated in a silver crucible for 5 hours at 175–180° in a closed tube. With exact control of the

[12] Paper XXII.

temperature, the reaction product was almost pure. A test sample, on cleavage with acid, gives a porphyrin which, when its ethereal solution is shaken with 2 per cent hydrochloric acid, does not color it at all. This is because of the insolubility of the hydrochloride; the cyano- and the phyllo-compounds would be extracted by the acid.

The potassium salt is difficulty soluble and precipitated well on dilution, while a little admixed cyanophyllin remained in the mother liquor. The yield of potassium erythrophyllin amounted to 0.6 g.

In order to isolate the erythrophyllin, 2.5 g. of finely powdered potassium salt were dissolved in alcohol and the carboxylic acid was transferred by the aid of primary phosphate to 4 l. of pure ether from which the alcohol was separated by copious washing. The beautiful, cherry red, ethereal solution required no further purification; after it was concentrated to 60 cc. a small quantity of decomposed product separated, then, after filtering and concentrating to 20 cc., there results an abundant crystallization of beautiful erythrophyllin in rhombic plates (1.2 g.). It was expedient to work up the mother liquor for the porphyrin.

From the Chlorophyll b Component.

Rubiphyllin.[13]

From isochlorophyllin *b* there is formed, only after a temperature of 150–155° has been attained, a very easily decomposable phyllin, which possesses a blue color and strong fluorescence in alcoholic potash and a green color in ethereal solution. The corresponding porphyrin is extracted from its reddish ethereal solution by 3 per cent hydrochloric acid with a deep blue green color.

The more beautiful and stable phyllin of the subsequent stage of the systematic decomposition was prepared in the pure state.

Methyl chlorophyllide *b* (0.7 g.) was dissolved in pyridine (2 cc.) and introduced into 7 cc. of boiling, methyl alcoholic potash; to bring about complete saponification it was heated for 5 minutes more over a small flame. The blood red, fluorescent solution was transferred, with the addition of 10 more cc. of lye, into a silver test-tube in order that it might be heated in the upright, closed tube for 3 hours at 165–170° in an oil bath. The potassium salt separated when the contents of the tube were diluted with an equal volume of water. This separation was more complete on salting out with potassium chloride.

[13] Paper XXII.

The dried, blue-violet lustered potassium rubiphyllin is dissolved in a little alcohol and suspended in the form of fine floccules by pouring the solution into 200 cc. of ether. The free phyllin is then easily brought into the ether, without the separation of difficultly soluble floccules, when acidified with monosodium phosphate. The solution has a violet tinged, red color and intense fluorescence. The alcohol is separated from it by repeated washing with water, in all about 10 l., and the volume of the solution is kept constant by replenishing it with alcohol-free ether. After drying with sodium sulphate the solution gives, when all except 1 or, at the most, 2 cc. has been quickly filtered off, a paste which consists of crystalline plates of pure rubiphyllin (0.5 g.).

Some potassium phyllophyllin is frequently already admixed with the crude rubiphyllin salt. In such cases, the two phyllins are separated by extracting their ethereal solution 2–3 times with 0.03 per cent ammonia. The rubiphyllin solution passes, without the formation of floccules and with a deep red color, into the ammonia, which is too dilute to react with phyllophyllin.

Rubiphyllin has also been prepared from the magnesium-free derivatives of chlorophyll *b* by Willstätter and Forsén's method and is easily obtainable in that way.

2 g. of methyl pheophorbide *b* are gradually introduced into 20 cc. of boiling, methyl alcoholic potash and heated with 2 g. of magnesium oxide in the sealed vessel for 3½ hours at 170°. Here, also, purification of the phyllin with very dilute ammonia is recommended. Its yield attains one half of the initial material.

4. Preparation of the Porphyrins.

From the Phyllins.

The phyllins are sensitive towards acids, they lose magnesium easily and form porphyrins which, however, if too quickly precipitated, may still contain ash. All phyllins can be quantitatively transformed into pure porphyrins by shaking their ethereal solutions with hydrochloric acid of moderate concentration; these, however, when diluted and neutralized, can be transferred into ether again only with difficulty. If it is only a question of rapid and complete separation in an ash-free condition, for example, of phylloporphyrin from calcium phyllophyllin, the hydrochloric acid liquor is simply neutralized rather

exactly, whereupon the porphyrin precipitates in microcrystalline floccules. A few examples are given to show how, in spite of their uncommonly slight solubility, the porphyrins are prepared as beautiful, pure crystalline materials.

Rhodoporphyrin.[14] Rhodophyllin forms a clear solution upon rapid, vigorous shaking with considerable glacial acetic acid, for example, 10–20 cc. for 5 or, at the most, 10 mg., and the porphyrin precipitates at once from the dark red fluid in the form of beautiful, glistening, crystalline plates, which mat upon the filter as a gray blue, silky shimmering network. If larger amounts are used the phyllin is much more easily dissolved in pyridine by gently warming and the resulting solution is slowly added to a large quantity of glacial acetic acid; the porphyrin quickly precipitates as small, lustrous, reddish brown rods.

Pyrroporphyrin[15] is easily obtained in glacial acetic acid from its phyllin since it is easily soluble in this solvent when hot, though it is characteristically distinguished from phylloporphyrin by its slight solubility in cold, glacial acetic acid. For example, 1.5 g. of pyrrophyllin (containing 15 per cent of combined ether) is dissolved, at room temperature or with gentle warming, in 80 cc. of glacial acetic acid. As soon as the solution has been filtered, the porphyrin begins to crystallize in plates that have a bright, blue gray, surface luster. The yield is almost quantitative (1.25 g.), but the preparations are wholly freed from ash by repeated crystallization from glacial acetic acid.

Erythroporphyrin.[16] Finely powdered erythroporphyrin (0.5 g.) is introduced into concentrated hydrochloric acid (100 cc.), which has been previously treated with a little ether. The magnesium-free acid that is formed is extracted from the clear, red solution by adding 10 l. of ether and 1 l. of alcohol and diluting with water. The large quantity of ether and the addition of alcohol are necessary since otherwise a portion of the porphyrin precipitates, even on transferring to ether. The bright red, ethereal solution can only be freed from alcohol, without crystallization taking place, by carefully allowing water to flow through it. Even on filtering, a portion can easily precipitate. When only moderately concentrated, the erythrophyllin crystallizes from the

[14] Ann. d. Chem. 358: 243. 1907 and 371: 91. 1909.
[15] Ann. d. Chem. 371: 95. 1909.
[16] Paper XXII.

ether in the form of silky lustered prisms. The filtrate has only feebly reddish color in consequence of the almost quantitative natur of this crystallization.

From the Magnesium-free Chlorophyll Derivatives.

Since the phyllins can be more easily purified than the porphyrins the procedure of introducing magnesium (by means of MgO), that is phyllin formation, is preferable even when working up pheophytin o phytochlorin to obtain porphyrins. Direct systematic decompositio to the porphyrins can be brought about also; for example, phyllopor phyrin[17] is formed from phytochlorin *e* by heating with methyl alco holic potash, even at as low a temperature as 140–150°, for a few hours

The weakly basic phytochlorins, on the other hand, produce pyrro porphyrin,[18] namely, chlorin *g* at 225–230°, and chlorin *f*, on rather long heating at 200°, after it has first changed into rhodoporphyrin a 140–150°.

The pyrroporphyrin separates as a difficultly soluble, potassium salt from the alkaline fluid; its hydrochloric acid number, 1½, and smaller solubility in cold, glacial acetic acid distinguish it from phyllo-porphyrin.

The transformation of derivatives of chlorophyll *b* into porphyrin is more difficult.

Phytorhodin *g* is unstable toward concentrated alkalies. It is transformed by them, even at the temperature of the water-bath and quickly at 140°, into an amorphous, brown substance, insoluble in ether. Porphyrin formation, which seems to be preceded by the reduction of an oxygen-containing group, is accomplished in the presence of much pyridine.

Phytorhodin *g* then produces phylloporphyrin very easily, even at a relatively low temperature.[19] 0.4 g. of phytorhodin *g* were dissolved in 10 cc. of pyridine and heated with 12 cc. of methyl alcoholic potash for 6 hours at 150–155°. After acidification of the potassium salt and extraction with ether only a few ether-insoluble floccules separated. In order to remove the more weakly basic admixtures, the porphyrin was extracted from the ether by means of 2 per cent hydrochloric acid and then again transferred into ether. When this was

[17] Ann. d. Chem. 371: 98. 1909 and 382: 183. 1911.
[18] Ann. d. Chem. 382: 183. 1911.
[19] Paper XXII.

concentrated, 0.25 g. of phylloporphyrin crystallized in the form of sharply defined, rhombic platelets.

Phytorhodins *k* and *i* produced pyrroporphyrin without any trouble, and even without the addition of pyridine, when they were heated 3 hours at about 200°.[20]

5. Description of the Phyllins.

The dicarboxylic acids ($MgN_4C_{31}H_{32}$) $(COOH)_2$.

Glaucophyllin[21]

crystallizes with a molecule of ether in bluish lustered, obliquely truncated prisms (Fig. 1 of Table V), which appear green in transmitted light under the microscope. The preparation, crystallized from the crude solution, dissolves considerably in dry ether and, in fact, much more abundantly than does rhodophyllin; it dissolves still more easily in moist ether, easily in acetone, considerably in alcohol though not easily. It is insoluble in chloroform and benzol.

Glaucophyllin, on the contrary, after recrystallizing or prolonged shaking with anhydrous ether, is insoluble in ether.

The freshly prepared glaucophyllin solution has a wonderful blue color and intense red fluorescence; glaucophyllin that has been treated with solvents and especially that which has been recrystallized dissolves with a violet color though this is still blue in comparison with that of rhodophyllin.

Glaucophyllin is extracted quantitatively with a violet color from an ethereal solution by means of a 0.01 per cent solution of sodium hydroxide; the solution alters on standing; 0.5 per cent disodium phosphate extracts it also with an initially violet, but soon turbid, red color which, on rather long standing, turns brown.

Cyanophyllin.[22]

It resembles glaucophyllin in the color of its solutions, which also gradually turn reddish in tinge. The free phyllin is unstable; it crystallizes in spindle-like forms.

In contradistinction to glaucophyllin it is not extracted from ether by 0.003 per cent ammonia; in contrast to erythrophyllin it is still

[20] Ann. d. Chem. 382: 194. 1911 and paper XXII.

[21] Ann. d. Chem. 371: 66. 1909.

[22] Paper XXII.

extracted by as low as 0.2 to 0.3 per cent disodium phosphate; it lies therefore, in acid properties between these isomers.

Rhodophyllin,[23]

which likewise contains closely bound ether, crystallizes in prisms These have irregularly inclined and shaped terminal domes and often assume whetstone and spindle-like forms (Fig. 2 of Table V). The behavior of the finely powdered material towards anhydrous ether is surprising; although a very small portion is temporarily dissolved the powder quickly changes into new, beautifully glistening, dark red sharply defined, rhombic crystals.

The solutions have a bluish tinged, red color resembling cherry juice, and show an intense, blood-red fluorescence.

Rhodophyllin is easily soluble in absolute alcohol (1 g. in 250 cc. when hot) and acetone, rather difficultly in methanol, still more difficultly in ether, insoluble in chloroform; on the other hand, it is very easily soluble in pyridine.

It is completely extracted from its ethereal solution by very dilute alkalies, for example, by 0.3 per cent disodium phosphate and by 0.01 per cent potassium hydroxide; 0.1 per cent potash dissolves rhodophyllin with a greenish tinged, brownish red color.

Potassium rhodophyllin. If an excess of methyl alcoholic potash is added to a hot alcoholic solution of rhodophyllin, the fluid brightens and its blue hue disappears, although the fluorescence does not change. The dipotassium salt then precipitates quickly in heavy, brightly shimmering crystals, prisms and little needles that have a blue color by reflected light; the liquor turns almost colorless. Cold, alcoholic solutions show the same reaction more slowly and it occurs even when they are greatly diluted. If the dry salt is triturated with methyl sulphate the dimethyl ester is formed. This crystallizes in violet prisms.

Erythrophyllin[24]

crystallizes from ether in acute, rhombic platelets; it is then only slightly soluble in ether, though easily soluble in absolute alcohol. Previous to its crystallization the ethereal solution is cherry red in color although much less blue than that of rhodophyllin. The crystals give a pure red, fluorescent solution without a blue tinge.

Erythrophyllin is distinctly less acid than rhodophyllin, since it is not extracted by 0.01 per cent potash lye; 0.1 per cent precipitates

[23] Ann. d. Chem. 358: 223. 1907 and 371: 71. 1909.
[24] Paper XXII.

potassium erythrophyllin. The dry, ethereal solution of rhodophyllin is precipitated by ammonia gas; erythrophyllin, however, remains dissolved as an ammonium salt. Nor is erythrophyllin precipitated by lime water.

The dimethyl ester forms red prisms, which are difficultly soluble in ether.

Rubiphyllin,[25]

upon its isolation, is very easily soluble in ether but, after recrystallization, it is insoluble in this solvent; it dissolves rather easily in alcohol. It forms triangular platelets and, more seldom, rhombs. The ethereal solution has a violet tinged, red color, less blue than that of rhodophyllin and not greenish tinged like that of phytorhodin. Pyridine, also, dissolves rubiphyllin with a yellowish red color, while rhodophyllin has a bluish red color in this solvent.

Rubiphyllin is extracted by 1 per cent, but not by 0.5 per cent, disodium phosphate and by 0.5 per cent ammonia, although not by 0.02 per cent. It is less acid than the other dibasic phyllins, even less so than erythrophyllin.

The monocarboxylic acids ($MgN_4C_{31}H_{33}$) ($COOH$).[26]

Pyrrophyllin.

It crystallizes in prisms (Fig. 3, Plate V), which are bounded at each end by paired domes. As a result of recrystallization it becomes very difficulty soluble in ether alone and remains easily soluble in alcohol and in acetone. It also dissolves easily in chloroform, carbon disulphide and warm benzol and is distinguished from the dicarboxylic phyllins in this way.

When shaken with 2 *N* sodium or potassium lye the ethereal solution loses its blue hue and alkali salts that are soluble in ether are formed. On the other hand, more dilute lye, for example 0.1 per cent sodium hydroxide, completely extracts the pyrrophyllin with an intensively red color, 0.01 per cent is colored only very little; a disodium phosphate solution does not react.

On shaking with *N* ammonia the salt crystallizes from the ether in bright red needles (as distinguished from phyllophyllin).

[25] Paper XXII.
[26] Paper VIII.

Calcium salt. When calcium chloride acts upon an ethereal solution of potassium pyrrophyllin, the calcium salt crystallizes in bright red needles. This dissolves in ether with much more difficulty than does the phyllophyllin salt, but it dissolves in alcohol more easily than does the latter, and especially easily in hot alcohol.

Phyllophyllin.

Its ethereal solution has a bluish tinged, red color and red fluorescence. It is very similar to its isomer in its chemical and physical properties but it loses its magnesium more easily. Toward alkalies it behaves like pyrrophyllin and, likewise, does not react with disodium phosphate. When acted upon by *N* ammonia the ammonium salt is divided between the aqueous and ethereal layers and, on salting out, passes entirely into the ether.

Methylester (See Chapter XIX).

Caesium phyllophyllin. The ethereal solution of phyllophyllin loses its blue hue when it forms a salt with caesium hydroxide; when strongly concentrated the caesium compound crystallizes beautifully. It is best obtained in larger quantities by mixing an ethereal phyllophyllin solution (obtained by concentrating the ether-soluble potassium salt) with more than an equal volume of alcohol in which caesium hydroxide has been dissolved. A large part of the caesium salt separates beautifully in bluish violet lustered, stout, often rectangular prisms (Fig. 4, Table V). The salt is not soluble in water; when once crystallized it is no longer soluble in ether. It is rather easily soluble in boiling alcohol and in chloroform.

Calcium phyllophyllin. The calcium salt, which is produced by shaking the ethereal solution with lime water or an ethereal solution of the potassium salt with calcium chloride, is best adapted for the isolation of phyllophyllin. The salt separates in dull violet floccules from its moderately concentrated solution when this is mixed with one fourth its volume of alcohol.

Calcium phyllophyllin dissolves very easily in chloroform (calcium phylloporphyrin only difficultly) and is precipitated by alcohol; 1 g. was dissolved in 30 cc. of chloroform and the solution diluted with 20 cc. of alcohol. The salt crystallized almost quantitatively in bright red needles.

By working rapidly it may be dissolved in absolute alcohol in small portions; it then soon separates again in beautiful needles, especially

quickly on warming, and in this new state it is insoluble in alcohol. The salt is also no longer soluble in ether after it has been once separated.

6. Description of the Porphyrins.

The distribution of the porphyrins between ether and hydrochloric acid, which is important for their recognition, has been described in sections 2 and 3 of Chapter XIV (hydrochloric acid number, distribution number).

Glaucoporphyrin.[27]

It forms reddish violet needles and can be recrystallized by dissolving it in pyridine and introducing the solution into a large quantity of hot, glacial acetic acid. It has a bluish tinged, red color in ether but is very slightly soluble in it; in its crystalline form it no longer dissolves in ether, alcohol or chloroform. A hydrochloric acid solution of porphyrin is violet; when dilute, the solution is more bluish violet and, when concentrated, dark reddish violet with a greenish tinge.

Cyanoporphyrin[28]

is formed, by the cleavage of phyllin by means of concentrated hydrochloric acid, in the form of a bluish green salt solution which turns pure blue when diluted with water. The porphyrin does not crystallize from its ethereal solution, which has a red color with a bluish tinge, until this has been concentrated. It crystallizes in the form of lustrous, reddish brown needles. These can be recrystallized from glacial acetic acid.

Rhodoporphyrin[29]

crystallizes from ether in two forms, in lustrous, reddish brown needles and small, dark steel blue, rectangular and hexagonal plates which are olive brown when viewed by transmitted light. When crystallized it is insoluble in the usual solvents. When its solution in hot, concentrated hydrochloric acid is diluted with 1/3 its volume of hot water, the hydrochloride crystallizes completely in slender needles.

[27] Paper VIII.
[28] Paper XXII.
[29] Papers V and VIII.

Upon the addition of alcoholic potash the porphyrin dissolves in alcohol with a red color, somewhat yellowish in tinge; the potassium salt crystallizes gradually in short, blue lustered prisms. Rhodoporphyrin dissolves also in dilute, aqueous alkalies (for example, in cold, 1 per cent sodium hydroxide) with a yellowish red color; on boiling, the sodium salt precipitates in extremely delicate, glistening crystals.

When warmed with acetic anhydride the porphyrin loses one molecule of water and forms a derivative which crystallizes in needles and small rhombic plates and which is very easily soluble in chloroform and acetone, even when these are cold.

Erythroporphyrin[30]

precipitates, even from dilute ethereal solutions, in silky lustered prisms and is insoluble in the usual solvents, except in pyridine and in acids. Twenty per cent hydrochloric acid, containing ether, easily dissolves it with a pure red color; the solution, it is true, has a blue tinge but this is much less pronounced than is the case with rhodoporphyrin.

Its basic properties, on account of the insolubility of the hydrochloride in dilute hydrochloric acid, can not be exactly compared with those of its isomers; its methyl ester, however, is easily soluble in hydrochloric acid, the hydrochloric acid number of this lying between 5 and 7.

Rubiporphyrin[31]

crystallizes in small, lustrous, reddish brown, rhombic, often rounded plates which are olive tinted brown by transmitted light. It is similar to rhodoporphyrin in its solubility as well as in that of its hydrochloride, which forms abruptly terminated prisms. The tint of the hydrochloric acid solution tends more to red than is the case with rhodoporphyrin.

The methyl ester, which crystallizes in violet lustered, obliquely terminated prisms, even from very much ether, gives a greenish red solution in glacial acetic acid when a drop of concentrated hydrochloric acid is added; the ester of rhodoporphyrin gives a violet red. The hydrochloric acid number of the ester of rubiporphyrin is 7½.

30 Paper XXII.
31 Paper XXII.

Phyllo- and Pyrroporphyrin.[32]

Both crystallize in dark red prisms with a violet, metallic luster; the crystals of the phyllo-compound have acute ends (Fig. 6 of Plate V), those of pyrroporphyrin are blunt (Fig. 5 of Plate V). The powders have brownish Bordeaux colors; that of phylloporphyrin is more bluish in tinge while that of pyrroporphyrin is more brownish tinted.

These substances dissolve very slightly in ether and in cold alcohol, though somewhat more so in hot alcohol. They are insoluble in benzol and carbon disulphide, rather easily in chloroform on boiling (pyrro- more easily), insoluble in cold chloroform and similarly in acetone. They are very easily soluble in pyridine.

Their behavior toward glacial acetic acid serves to distinguish them since phylloporphyrin dissolves very easily with a dark violet red color and strong violet tinge, which does not alter on warming; pyrroporphyrin, however, dissolves in glacial acetic acid with considerably greater difficulty and crystallizes from the hot solution.

The fluorescence of the two porphyrins in ether is but small in comparison with that of the phyllins; with pyrro-, however, it is quite marked, and with phyllo- very slight. These solutions are brownish red, with a less bluish tinge than in the case of rhodo- and glaucoporphyrins; the color of pyrroporphyrin is browner than that of phylloporphyrin.

Since the hydrochloric acid number of phylloporphyrin is ¾ and that of pyrroporphyrin, 1½, 1 per cent acid extracts phylloporphyrin almost completely from its ethereal solution while in the case of pyrroporphyrin less than one half is extracted.

The hydrochlorides show a great difference in their solubilities; the phylloporphyrin salt is easily soluble in hydrochloric acid, that of pyrroporphyrin is insoluble in moderately dilute hydrochloric acid.

The solution of phylloporphyrin in 6 per cent hydrochloric acid shows, with increasing concentration, the colors, blue violet, red violet and then a brownish red with a greenish tinge. In hydrochloric acid pyrroporphyrin is red with only a slight bluish tinge, in greater concentrations it is much more bluish red and tinged blue.

Both porphyrins dye animal fibers beautifully and permanently. Very dilute hydrochloric acid solutions dye silk with essentially dif-

[32] Paper VIII, see also Ann. d. Chem. 382: 184. 1911.

Phyllins	Glaucophyllin	Cyanophyllin	Rhodophyllin	Erythrophyllin	Rubiphyllin	Pyrrophyllin	Phyllophyllin
Color in ether	blue	blue	bluish red	red	violet tinged red	bluish tinged red	bluish tinged red
In chloroform	insoluble					soluble	
By 0.1 N ammonia	is completely extracted from ether					insoluble	
Solubility of the ammonium salt in ether	insoluble			soluble	insoluble	insoluble	soluble
Behavior towards disodium phosphate	is extracted from ether by 0.3 per cent.			0.3% is colored very little, goes abundantly into 0.5%, completely into 1%	is not extracted by 0.5% but is extracted by 1%	insoluble	
Porphyrins	**Glauco-porphyrin**	**Cyano-porphyrin**	**Rhodo-porphyrin**	**Erythro-porphyrin**	**Rubi-porphyrin**	**Pyrro-porphyrin**	**Phyllo-porphyrin**
Color in ether	bluish tinged red		brownish red		light red	red	brownish red
Color in HCl in 20%	red violet	blue green	red violet	pure red	strongly reddish tinged blue	bluish red	brownish red
Color in HCl in 4%	blue violet	blue	bluish red	violet red	bluish red	bluish red	violet red
Solubility of the hydrochloride in dilute HCl	easily soluble		difficultly soluble	insoluble	difficultly soluble	difficultly soluble	easily soluble
HCl number	3½	4	3	—	4½	1½	¾

ferent colors, namely, pyrroporphyrin with a copper red color and phylloporphyrin strongly dichroic; the reflected light, when looking across the fabric, is bronze in color; when looking directly down upon it, the color is greenish bronze and copper red.

The monobasic porphyrins react quantitatively, even with very dilute alkalies. The total pigment is immediately extracted from ether by 0.01 per cent caustic soda and potash solutions. Solutions are formed that are clear, yellowish tinted red by transmitted light; by reflected light, on shaking, the liquor is observed to be full of extremely small particles. The addition of stronger lye increases the precipitation, distinctly brownish yellow needles can then be recognized under the microscope.

The methyl esters of both porphyrins, formed by means of methanol and hydrochloric acid, are somewhat more weakly basic than are the carboxylic acids; the hydrochloric acid number of the phyllo-compound is 1–1¼, that of the pyrro-derivative is 2½. The esters are much more easily soluble than the free porphyrins, namely, very easily in chloroform and glacial acetic acid, easily in acetone, more difficultly in ether. The phylloporphyrin ester crystallizes from ether in reddish violet lustered, rhombic plates; the pyrro-ester crystallizes in long prisms.

Comparison of Acid Properties by the Ammonia Method.[33]

The porphyrins, like the phyllins, are distinguished by means of their acid properties; rubi- and erythroporphyrin are weaker acids than rhodoporphyrin.

The action of ammonia gas upon the finely powdered acids, after they have been dried to constant weight, offers a means for the determination of the acid groups and for a comparison of the acid properties. Characteristic differences are often shown when the ammonium salts that are formed dissociate under ordinary and under diminished pressure.

Rhodo-, erythro- and rubiporphyrin take up two molecules of ammonia. Rubiporphyrin, under ordinary pressure, slowly gives up all the ammonia again when placed in a phosphorus pentoxide desiccator; this happens even in one day in the vacuum produced by a water jet pump. Erythroporphyrin, also, in an evacuated desiccator,

[33] Paper XXII; see also, for example, Ann. d. Chem. 382: 174 and 179. 1911; and Ann. d. Chem. 387: 366 and following pages. 1911.

quickly loses the two molecules of ammonia; on the other hand, only one molecule of ammonia dissociates from rhodoporphyrin in an ordinary vacuum and the second molecule is given up only extremely slowly in a high vacuum.

Absorption Spectra.[34]

Descriptions and representations of the absorption spectra are given here in the same way, as, for example, in Chapter VI and in Plates VI and VII. Quite weak shadows (|) were not considered in the case

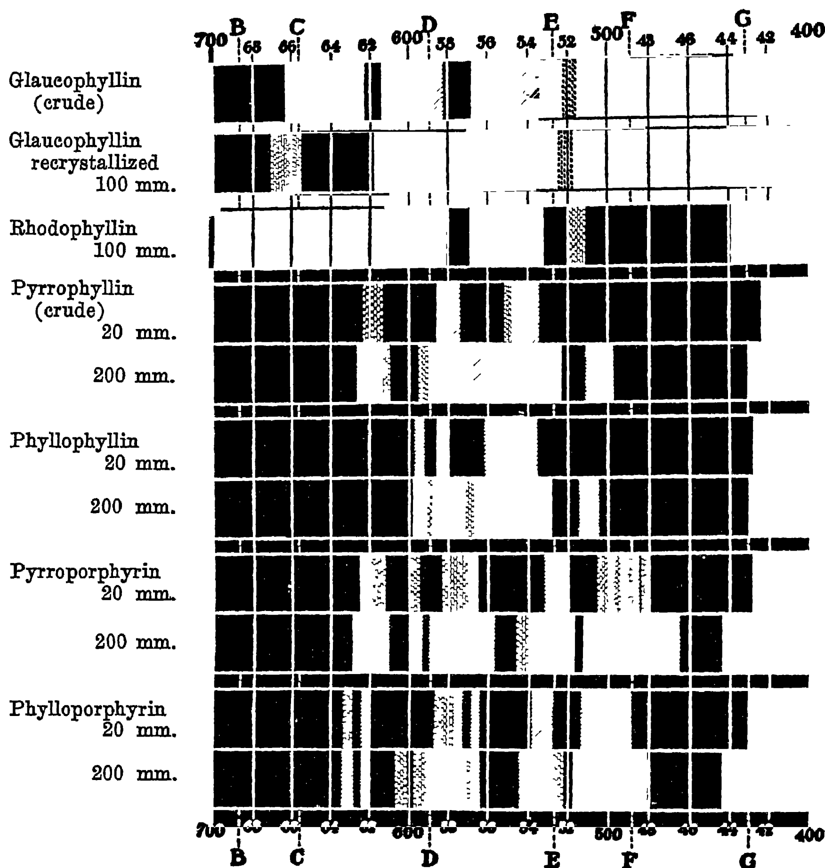


Fig. 15.

of the following compounds. The measurements were made with a prism spectroscope but in the diagram (Fig. 15) the intervals between

[34] Papers V and VIII.

individual wave-lengths have been made equal; *i.e.*, as they appear in a grating spectroscope. The graphically represented spectra of recrystallized glaucophyllin and of rhodophyllin were measured in alcoholic solution. All others have been measured in ethereal solutions.

Glaucophyllin.

An ethereal solution of the carboxylic acid, of unknown concentration, was prepared from the crude, freshly isolated, potassium salt.

The absorption spectrum shows a very great difference, in comparison with that of the recrystallized substance. Band I is much more intense and is the strongest; it is very sharply defined and so wide that it extends far into the orange. It is quite perceptible even in extremely dilute solutions. This absorption in the red is the chief difference between glaucophyllin and rhodophyllin.

Thickness of layer, in mm.	2.5	10	40
Band I	652— —633	655—630	662.5—622.5
" II	605 . . . 594.5	607.5—594.5	613—587— —582
" III	559 . 551	561— —549	547—542— —536
" IV	—	—	521 . . 516
End absorption	—	434—	437—

The order, arranged according to intensity, is: I, II, III, IV.

The absorption spectrum of the recrystallized material, especially in the case of a thin layer, resembles that of rhodophyllin much more than it does that of crude glaucophyllin; yet, recrystallized glaucophyllin is distinguished from rhodophyllin, when the thickness of the layer is rather great, by the appearance of a new absorption band in

0.1 G. OF RECRYSTALLIZED GLAUCOPHYLLIN IN 5 L. ALCOHOL.

Thickness of layer, in mm.	5	20	100
Band I	—	—	670 . . . 655
" II	605 . . . 597	610—597 . . . 594.5	617—587— —585
" III	557 . . 551	563— —549	570—536
" IV	—	—	523.5 . . 517.5
End absorption	430.5—	435—	440—

the red at line C. In addition, band IV in the green is weaker than band III of rhodophyllin.

The order, arranged according to intensity, is: II, III, I, IV.

Rhodophyllin.

The absorption spectrum of this phyllin is simple and reminds one very much of hemin; it consists in the visible region of the spectrum of two, strong, sharply defined bands in the orange to yellow and in the green, which are followed by a much weaker band in the green and a strong end absorption in the violet. The chief distinction between rhodophyllin and hemin consists in the band in the orange in the case of hemin being shifted toward the right so as to include almost the whole yellow region.

0.1 G. RHODOPHYLLIN IN 5 L. ALCOHOL.

Thickness of layer, in mm.	2.5	20	100
Band I	602 . . . 596	605—592 . . 588	610—581
" II	554 . 553	562—545	568—532
" III	—	—	520 . 512
End absorption	424—	431—	438—

The order, arranged according to intensity, is: I, II, III.

Pyrrophyllin

may be distinguished from rhodophyllin by less light absorption in the orange. The characteristic orange band of rhodophyllin appears, in thin layers of solution, to be shoved wholly into the yellow as a very sharply defined, narrow band. The band that follows this in the green region and which is very similar to band II of rhodophyllin, is broader and less sharply defined.

Only in a thicker layer does a narrow, weak absorption band (I) appear and this, in fact, is further toward the red than is the case with rhodophyllin. The orange band of the latter is then split into two bands, into a weak one shifted toward the left and the intense band (II) which embraces the yellow.

Pyrrophyllin, like glaucophyllin, is altered when recrystallized. Recrystallized phyllin permits the passage of more red light than does

the crude product; the absorption band in the red (I) becomes very faint.

PYRROPHYLLIN FRESHLY ISOLATED FROM 0.1 G. POTASSIUM SALT IN 5 L. ETHER.

Thickness of layer, in mm.	20	100
Band I	622.5 . . 614	624— —613
" II	585—578— —574	587—571.5
" III	551 . . . 548—540— —534.5	561— —556—526.5
" IV	—	509 . . 498
End absorption	422.5—	425—

The order, arranged according to intensity, is: II, III, I, IV.

0.1 G. OF RECRYSTALLIZED PYRROPHYLLIN IN 5 L. OF ALCOHOL.

Thickness of layer, in mm.	10	100	200
Band I	—	—	621 . . 618
" II	584—579— —572	587—569.5	} 589—567 . . . 561
" III	545— —538	{ 559— —555— 528— —526	} 561—522
" IV	—	508 . . . 498	509 . . . 496 . 490
End absorption	421—	424—	427.5—

The order, arranged according to intensity, is: II, III, IV, I.

Phyllophyllin.

The ethereal solution of phyllophyllin was freshly prepared from pure, recrystallized, calcium salt by careful acidification.

The absorption before the Fraunhofer line G consists of 5 bands: I is in the orange; II, the most intense, is in the yellow; III and IV, in the green; a very much weaker band in the blue follows these. Bands III and IV correspond to bands II and III of rhodophyllin. Band I of rhodophyllin in the orange is split into two, sharply defined, narrow bands, the second of which, standing closely to the right of the sodium line, is very dark. Even in greater concentrations, the passage of light is permitted through the green near the E line, as in the case of rhodophyllin.

Phyllophyllin is distinguished from freshly isolated pyrrophyllin by the fact that band I is shifted much farther towards the yellow and that band II is narrower.

0.1 G. IN 5 L. OF ETHER.

Thickness of layer, in mm.	5	100	500
Band I	595 . . . 592	596— —591.5	602—525 . . . 519 (Bands I–III)
" II	584.5— —581	586—580 . . 572	
" III	561 . . . 542 . . 539	563—534 . 529	
" IV	—	514 . 506	519—497
" V	—	—	484.5 . . 475
End absorption	425 —	429.5—	435—

Even when the phyllophyllin solution stands for a short time, an initial decomposition is betrayed by the appearance of the characteristic double bands of phylloporphyrin at $\lambda = 633$—627 and 625 to 618.

Pyrroporphyrin and Phylloporphyrin.

The absorption spectra of the two porphyrins consist of, not mentioning the end absorption, 6 bands, which appear as absorption maxima in the orange, the green and the blue.

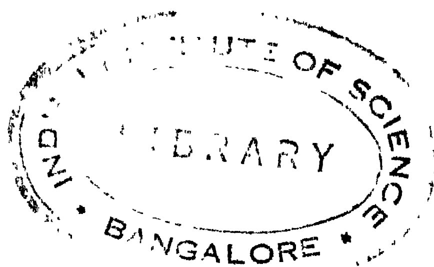
Pyrroporphyrin is distinguished from the phyllo- compound by the fact that, for it, band I is shifted toward the red end and is much weaker. Furthermore, bands II and III are more intense in the case of pyrroporphyrin; the former is located in the same position while the latter is shifted towards the violet. The separate absorption bands, IV and V, of phylloporphyrin are united into a single band, IV, in the case of pyrroporphyrin, in such a way that it is accompanied on the left side by a shadow. Bands V and VI of pyrroporphyrin are shifted farther towards the violet than the corresponding bands of phylloporphyrin and band VI is more complicatedly membered than the corresponding band VII of phylloporphyrin. It is distinctly ribbed so that 3 dark bands stand out prominently.

0.1 G. PYRROPORPHYRIN IN 5 L. OF ETHER.

Thickness of layer, in mm.	20	500
Band I	—	652 . 648.5
" II	624—621 . 613	636 . . 630—609 . . 600.5
" III	599 . . 594.5	600.5—594 . . . 590
" IV	583 . . . 574 . . 570—565	590—556
" V	531.5— —526.5—523.5 . . . 520.5	547 . . 541— (Band V to End absorption)
" VI	505 . . . 499.5— —497 . . . 494— — 490.5 . . . 488.5— —485.5 . . . 481	
End absorption	428—	

0.1 G. PHYLLOPORPHYRIN IN 5 L. OF ETHER.

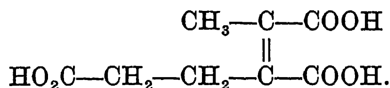
Thickness of layer, in mm.	20	200
Band I	633 .. 630	633.5— —629.5
" II	624— —620.5	624—620
" III	—	606 .. 602
" IV	587 .. 577— —574	} 597.5 ... 589.5—572 .. 569
" V	568.5 ... 565.5	} 569— —565
" VI	538 ... 528	543.5— —536—528 ... 523.5
" VII	{ 513— —505 { 505—496 ... 489	517—484 ... 481.5
End absorption	430.5—	443.5—


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XXI. OXIDATION OF THE CHLOROPHYLL DERIVATIVES.[1]

William Küster[2] investigated the oxidation of hemin in his fundamental work on the pigment of the blood. The oxidation leads to the imid, $C_8H_9O_4N$, of the tribasic hematic acid:



The literature contained only a single statement regarding the oxidation of chlorophyll. L. Marchlewski[3] had oxidized phylloporphyrin with chromic acid by using the Küster procedure and obtained hematic acid in its N-free form ($C_8H_8O_5$) from it. This result appeared to support the idea of a relationship between the basic cleavage products of chlorophyll and hemin.

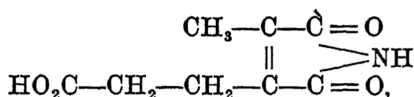
The supposition, however, that chlorophyll behaves the same as hemin upon oxidation has proved false. An investigation that was carried out by Willstätter and Asahina by subjecting a series of chlorophyll derivatives to different methods of oxidation showed the distinctive difference between them.

The initial materials were, in particular, phylloporphyrin, pyrroporphyrin, rhodoporphyrin and phytochlorin. Oxidation gave the same result in all cases. When lead peroxide and sulphuric acid, chromic acid or Caro's acid are used, the oxidation produces a mixture which, if small cleavage fractions of the molecule such as acetic acid and carbon dioxide are disregarded, consists of two principal products; namely, hematic acid, which always appears as an imid of the formula,

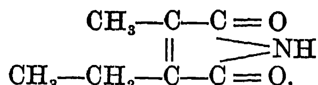
[1] Paper IX.

[2] Beiträge zur Kenntnis des Hämatins, Tübingen 1896. Zeitschr. f. physiol. Chem. 28: 1. 1899; 29: 185. 1900; 44: 391. 1905; 54: 501. 1908; 61: 164. 1909. Ann. d. Chem. 315: 174. 1900. See also W. Küster, Das Hämatin und seine Abbauprodukte in Abderhalden's Handbuch der biochemischen Arbeitsmethoden. Bd. II, 628. 1910.

[3] Bull. de l'Acad. des Sciences de Cracovie, 1902. 1.



and methylethylmaleic imid,



Küster first obtained this maleic imid by cleavage of carbon dioxide from the imid of hematic acid and then prepared it synthetically;[4] it never appeared in the oxidation of hemin.

Oxidation of the chlorophyll derivatives takes place most smoothly when chromic acid is used. Küster oxidized hematin in a warm acetic acid solution. It is much more advantageous to allow the chromic acid to act in a strong, sulphuric acid solution at a low temperature. This method is also preferable for hemin. Hemin itself may be used then and the isolation of hematin and the wearisome expulsion of the acetic acid, which Küster prescribes, avoided. The oxidation product, whose isolation is very simply accomplished, does not lose ammonia and is characterized by its purity.

The yields of oxidation products are important in furthering our knowledge of the structure of the compounds mentioned. Küster, on the basis of his numerous determinations of the yield of hematic acid, assumed that three, or perhaps even four, molecules of hematic acid were formed from as many pyrrol nuclei of hemin. According to Küster, hematoporphyrin furnishes 60.5 per cent of crude hematic acid, or 58 per cent of pure; calculated for 2 mols., 61.2 per cent; hemin produces 70 per cent of crude hematic acid or 60 per cent of pure; calculated for 2 mols., 56.2 per cent.

O. Piloty,[5] on the other hand, concluded from his beautiful work on the reduction of hemin that only two molecules of hematic acid are formed, since they are formed from the hemopyrrol carboxylic acid that he discovered.

The investigations of Willstätter and Asahina, which, certainly as regards the hemin considered for purposes of comparison, form only a supplement to the comprehensive, experimental data of Küster, sup-

[4] Ann. d. Chem. 345: 1. 1905.

[5] Ann. d. Chem. 366: 237. 1909.

ported the interpretation of Piloty. The yield of crude hematic acid is increased by admixtures; in its purification the yields are reduced to such an extent that they no longer amount to the theoretical value for 2 molecules.

The porphyrins from chlorophyll furnish approximately one molecule of hematic acid. On the other hand, the yield of methylethylmaleic imid probably amounts to more than one molecule; figuring in the losses on isolation, the yield amounts even to about 1½ molecules, so that we have to assume that the maleic imid is formed from two pyrrol nuclei.

Accordingly, the difference between the porphyrins derived from chlorophyll and those from hemin extends to at least 2 of their 4 pyrrol nuclei.

Oxidation of Phylloporphyrin with Lead Dioxide.

The oxidation is carried out advantageously with small portions.

One g. of phylloporphyrin is dissolved in 20 cc. of 50 per cent sulphuric acid and diluted with 30 cc. of water. While stirring with a motor and cooling with ice, 20 g. of lead peroxide are introduced, in small portions, during the course of a half hour. The solution loses its color even before all the oxidizing agent has been added; the stirring is continued for another hour and the solution allowed to stand over night in the ice chest. The lead sludge is then removed by filtration and washed with water (50 cc.). The yellowish filtrate, together with the wash water, is thoroughly extracted with ether and the ethereal solution dried. When evaporated it leaves a brownish syrup.

8.4 g. of the crude oxidation product were obtained from 20 g. of phylloporphyrin. This had been, in part, worked up in rather large portions in a series of experiments, each with 1 g.; the yield amounted to half of the porphyrin.

The oxidation product consists of a neutral and an acid portion. In order to separate these, the yields from 5 experiments were united and dissolved in a little water; when this was done a small quantity of insoluble, fatty product remained. The filtrate was made feebly alkaline with soda and extracted with ether. After evaporation of the ethereal solution an almost colorless, drusy-crystalline product remained; as a consequence of its volatility at the temperature of the water bath long needles occasionally sublimed in the neck of the flask.

The alkaline soda, mother liquor was acidified and then saturated with ammonium sulphate; the acid, which even when in an impure state formed characteristic rosettes of small, delicate needles, was isolated by extraction with ether.

This separation yielded 3.3 g. of neutral, and somewhat more than 2 g. of acid, oxidation product.

The neutral substance is completely purified by recrystallizing twice from water, in which it is rather easily soluble when warm and difficultly soluble when cold. It dissolves very easily in organic solvents except the petroleum hydrocarbons. It agrees exactly with Küster's description of methylethylmaleic imid as regards its melting point of 67–68° C., its weak, iodoform-like smell, its initially sweet, and finally bitter, taste and its other characteristics.

The acid oxidation product of phylloporphyrin proves to be identical with real hematic acid; *i.e.*, with the "Imid der dreibasischen Hämatinsäure" of the formula, $C_8H_9O_4N$, which Küster discovered and carefully distinguished. The acid was very easily soluble in water and alcohols; it was obtained in a sufficient state of purity as stellately grouped, hard needles by recrystallizing once from ethyl acetate and then from an ether-benzol mixture. The melting point was found to be 112–113°, depending somewhat upon the manner of heating.

Oxidation of Phylloporphyrin with Cold Chromic-Sulphuric Acids.

5 g. of phylloporphyrin are dissolved by stirring in a mortar with 70–90 cc. of 50 per cent sulphuric acid and then diluted with 30–50 cc. of water. After the deep red solution has been cooled to 0° C., and while it is being stirred with a turbine, the chromic acid (13 g. in 50 cc. of water) is dropped into it by means of a Frankenstein stirrer, the temperature not being permitted to rise above 5–7° C. during the procedure. Introduction of the chromic acid requires an hour; without much foaming the fluid assumes a dark, wine-red color and then turns olive green. The stirring is continued for another hour and the liquor, which has meanwhile turned pure green in color, is not filtered till the next day from the small quantity of undissolved, reddish brown material (at most, 0.2 g.) which has escaped oxidation. Without dilution, the filtrate is then exhaustively extracted with ether. The ethereal solution, when dried with sodium sulphate, leaves, on evaporation, in the form of a faintly yellowish syrup, a mixture of

the two oxidation products which, when placed in a vacuum over soda lime, slowly loses the smell of acetic acid.

The mixture does not crystallize but it is resolved into its two components by solution in water, neutralization, and extraction with ether from the soda alkali. The solution then forms the two components on acidification and saturation with ammonium sulphate; both oxidation products crystallize at once.

In experiments, each made with 5 g. of phylloporphyrin which had been purified by fractionation with hydrochloric acid and, in addition, for experiment III, recrystallized from ether, the following yields were attained:

	Crude mixture	$C_7H_9O_2N$	$C_8H_9O_4N$
Found I	3.55	1.35	1.00
" II	3.30	1.67	1.31
" III	3.00	1.20	1.10
Theoretical yield for each molecule	3.18	1.37	1.81

In order to ascertain the accuracy of our determination of the yield and the unavoidable losses in the separation we

1. Dissolved weighed amounts of imid, as well as of hematic acid, in 100 cc. of 35 per cent sulphuric acid and recovered them precisely according to our method of isolating the crude product of the oxidation.

Used 0.890 g. of imid; found 0.842 g. (94.7%).
" 1.000 g. of hematic acid; found 0.897 g. (89.7%).

2. The two pure oxidation products were mixed and separated according to the method described.

Used 1.365 g. $C_7H_9O_2N$ + 1.386 g. $C_8H_9O_4N$; found 1.278; *i.e.*, 93.6 per cent imid and 1.387; *i.e.*, 100.0 per cent hematic acid.

Since pure ether was used here (not that which had been recovered from the isolation of the imid and which, therefore, contained imid), some of the imid was lost by volatization with the ether vapor.

If the losses, determined here for the double isolation, are added to the yields obtained in experiment II, the following estimates are obtained for the two oxidation products:

Imid	38 instead of 27% = 1 molecule
Hematic acid	28 instead of 36% = 1 molecule.

Pyrroporphyrin and rhodoporphyrin, upon oxidation, gave the same result as phylloporphyrin.

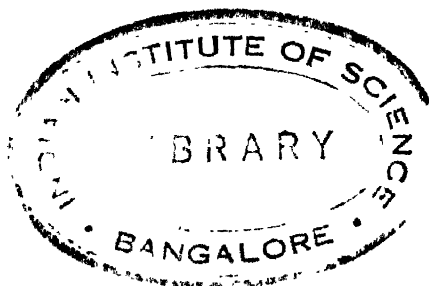
Oxidation of Hemin.

With lead dioxide. Hemin, in portions of 1 g., was dissolved in 15 g. of concentrated sulphuric acid while being stirred in a mortar; the fluid was introduced, drop by drop, while cooling with ice and stirring, into a suspension of 15 g. of lead peroxide in 60 g. of 50 per cent sulphuric acid. The oxidation was carried to completion just as was done with phylloporphyrin; 5 g. of hemin yielded 2.1 g. of the crystallized, crude product and, after purifying with soda solution in which process a small amount of non-crystallizable, waxy-like material remained undissolved, 1.8 g. of rather pure hematic acid.

With chromic-sulfuric acid. Purer hematic acid was obtained as a result of the oxidation with chromic-sulphuric acids than by oxidation in warm, glacial acetic acid according to Küster's method and the yield of crude product was lower, perhaps as a consequence of the absence of admixture.

5 g. of hemin are stirred with 25 g. of concentrated sulphuric acid and diluted with 50 cc. of 50 per cent sulphuric acid. The turbid, dark brown fluid is cooled with ice, and the aqueous, chromic acid (15 g. in 50 cc.) introduced, drop by drop, while stirring. The temperature is maintained at 5–7° during the hour required for introducing the solution. The color changes to olive green and, on standing a few hours, turns pure green; the precipitate to be filtered off is only about 0.25 g.

The yield in crude imid of the tribasic hematic acid amounted to 2.34 g. in repeated experiments; in purifying (according to the method of Küster), 1/15th of the quantity of calcium carbonate required for salt formation was sufficient. The pure, re-isolated hematic acid amounted to 1.56 g.



XXII. REDUCTION OF THE CHLOROPHYLL DERIVATIVES.[1]

1. Historical.

M. Nencki and J. Zaleski[2] discovered hemopyrrol when they reduced hemin with hydrogen iodide and phosphonium iodide. From analyses of the crystallized picrate and of the amorphous compound with mercuric chloride they derived the formula, $C_8H_{13}N$, for the base. They also succeeded in splitting off hemopyrrol from a chlorophyll derivative, a result that could be expected from the relations between hematoporphyrin and the phylloporphyrin found by Hoppe-Seyler and E. Schunck. Nencki, in fact, jointly with L. Marchlewski,[3] observed the formation of hemopyrrol in the reduction of the so-called phyllocyanin copper acetate, and analyzed the base in the form of its mercury compound.

Nencki died a few months after the publication of his discovery and since then, hemopyrrol, although easily accessible by the method of Nencki and Zaleski, and important for a knowledge of the blood and leaf pigments, has been until recently only insufficiently investigated.

The investigations of W. Küster have, indeed, thrown light on the nature of the side chains in hemopyrrol. Although Nencki and Zaleski had spoken of a butyl or a propyl group, W. Küster,[4] as a result of its oxidation to methylethylmaleic imid, made it appear very probable that hemopyrrol is an α-methyl-β, β-methylethyl pyrrol. In addition to this Küster showed that hemopyrrol originates from a different nucleus of the hemin than does hematic acid, for its formation occurs without the splitting off of CO_2.

According to Küster, hemopyrrol is not a single pure substance. By means of mineral acids he separated the mixture into an acid and a basic fraction. The former contained pyrrol, the latter probably the corresponding pyrrolin.

[1] Paper XVIII.

[2] Ber. d. d. Chem. Ges. 34: 997. 1901.

[3] Ber. d. d. Chem. Ges. 34: 1687. 1901.

[4] Ann. d. Chem. 346: 1. 1906 and Zeitschr. f. Physiol. Chem. 55: 526. 1908.

In an exhaustive investigation O. Piloty[5] then undertook to prepare hemopyrrol in a pure state. He carried out the reduction with tin and hydrochloric acid. He sought to separate the pyrrol from the hydrogenated base by fractional distillation in a vacuum. His hemopyrrol crystallizes partially, the melting point is 39°; the picrate always has a melting point of 108.5°. Piloty completed the proof of its constitution by oxidizing the hemopyrrol with nitrous acid to the oxime of methylethylmaleic imid (M. P. 201°).

In order to compare the products of the cleavage of chlorophyll and hemin by reduction, Willstätter and Asahina conducted an investigation which showed that the hemopyrrol from chlorophyll and that from hemin are the same but that it is not, as had till then been generally assumed, a pure pyrrol, but a complicated mixture of pyrrol homologues.

The admixed, hydrogenated, pyrrol bases, secondary products of the reduction, could be easily separated quantitatively by extraction with monosodium phosphate. But there was no method for resolving the thus purified hemopyrrol into its constituents. Fractional distillation in vacuum offered no prospect of success. An attempt was made to effect a separation by fractional crystallization of the picrates, but without good results. Willstätter and Asahina, however, found a good method which consisted of fractional salt formation with picric acid, and, by repeated application of the method, isolated three components of the hemopyrrol. Two of these were pure (isohemopyrrol and phyllopyrrol), a third (hemopyrrol) has not yet been shown to be a single, pure substance.

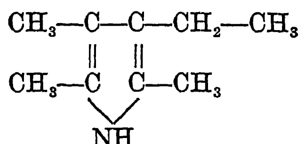
One constituent of the hemopyrrol mixture corresponded approximately and best to the known data: melting point of the picrate, 108–109°; of the methylethylmaleic imid oxime that is formed by the action of nitrous acid, 201°; the name, hemopyrrol was, therefore, retained for this component.

A second base, whose picrate melted at 119°, was obtained in a pure, crystalline condition, melting at 16–17°; it behaved toward nitrous acid as does hemopyrrol and gave the second oxime of methylethylmaleic imid (M. P. 129°). For this new component Willstätter and Asahina introduced the name, isohemopyrrol, while H. Fischer and E. Bartholomäus,[6] who isolated it about the same time, called it hemopyrrol.

[5] Ann. d. Chem. 366: 237. 1909. Ann. d. Chem. 377: 314. 1910. O. Piloty and E. Quitmann. Ber. d. d. Chem. Ges. 42: 4693. 1909.

[6] Ber. d. d. Chem. Ges. 44: 3313. 1911.

Most important was the discovery of a third differently constituted component, the phyllopyrrol of Willstätter and Asahina, which lacked all the specific characteristics of hemopyrrol. It contained one more carbon atom and was explained as a trimethylethylpyrrol, corresponding to the formula:



H. Fischer and E. Bartholomäus also observed this component at the same time.

The salt of phyllopyrrol with picric acid is more difficultly obtained because it is the most easily soluble of the picrates. Oxidation with chromic acid—the reaction with nitrous acid does not proceed smoothly—does not produce the imid of methylethylmaleic acid. Phyllopyrrol is distinguished from the pyrrol bases with 8 carbon atoms principally by the absence of the pine splinter reaction and of the color reaction with dimethylaminobenzaldehyde that was discovered by P. Ehrlich[7] and explained by O. Neubauer.[8] Nor is it precipitated in an acid solution by diazonium salt.

Yet the base cannot be anything else than a pyrrol. For it took up, when reduced, four atoms of hydrogen and produced a saturated hydro-derivative. The same was—notwithstanding all the differences of the parent pyrrol—extremely similar to the hemopyrrolidins that were prepared for purposes of comparison. If the crystalline base were a hexahydromethylindole it could take up only two atoms of hydrogen.

The differences between hemo- and phyllopyrrol are explained by the fact that all four carbon atoms in the latter have side chains.

The pine shaving reaction is, analogously to the dimethylaminobenzaldehyde reaction, to be understood as a condensation of the aldehydes of the wood with the pyrrol nuclei. F. Feist[9] has shown that pyrrols, substituted at all four carbon atoms, can not condense with aldehydes and H. Fischer[10] has noticed that pyrrols similarly substituted do not give the Ehrlich reaction.

[7] Die Medizinische Woche. 1901: 151.

[8] Verhandlg. d. Ges. d. Naturf. u. Ärzte. 1903, Part II, 2nd half, 68.

[9] Ber. d. d. Chem. Ges. 35: 1647. 1902.

[10] Zeitschr. f. Physiol. Chem. 73: 204. 1911.

Phyllopyrrol shows another especially interesting difference from hemopyrrol. This difference is contrary to statements occurring in the literature. It is not precipitated by an aqueous, mercuric chloride solution; consequently it always remained in the mother liquor in the separation of hemopyrrol by Nencki and Zaleski's[11] method.

The tri-substituted pyrrols are precipitated by mercuric chloride; the tetra-substituted are not. The reaction of pyrrols with mercuric chloride, accordingly, is not as has been assumed, a salt formation at the nitrogen (phyllopyrrol, of course, gives a salt with potash) but probably a mercurization at the carbon, such as was first observed by J. Volhard[12] in the case of thiophene and by O. Dimroth[13] and other experimenters in the case of many aromatic compounds.

The mixture of different pyrrols is always formed when derivatives of chlorophyll are reduced with hydriodic acid and phosphonium iodine, or with tin and hydrochloric acid, as well as when hemin is reduced by Nencki and Zaleski's method, or hematoporphyrin by Piloty's method; in those cases also, therefore, where the formation of volatile bases can be attributed to only two nuclei of the pigment molecule, or perhaps even to only one.

Of the chlorophyll derivatives investigated, phylloporphyrin produced the largest yield of volatile reduction products since it contains only one carboxyl group. The following concept may be formed of the rôle played by the four, nitrogen-containing nuclei of phylloporphyrin during oxidation and reduction: two nuclei, on reduction, give tri-substituted pyrrols. They are the ones from which methylethylmaleic imid is formed by oxidation. One nucleus, after reduction, appears as phyllopyrrol; it is probably the same one that is lost as a result of oxidation. The fourth nucleus, in reduction as in oxidation, retains its carboxyl; it, therefore, does not form a volatile pyrrol derivative; its oxidation product is the imid of hematic acid.

These results did not complete the explanation of hemopyrrol, but continuation of the investigation by Willstätter and Asahina was outstripped by the rapid publications of other investigators.

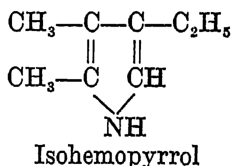
[11] Ber. d. d. Chem. Ges. 34: 1003. 1901.

[12] Ann. d. Chem. 267: 172. 1891.

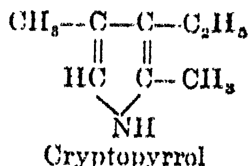
[13] Ber. d. d. Chem. Ges. 31: 2154. 1898; 32: 758. 1899; 35: 2032, 2853. 1902.

L. Knorr and K. Hess,[14] even at that time, had synthesized 2. 4-dimethyl-3-ethylpyrrol and showed it to be different from the pyrrols obtained by the reduction of hemin. This pyrrol homolog was discovered soon afterwards by H. Fischer and E. Bartholomäus[15] as a constituent of hemopyrrol and called cryptopyrrol.

The three bases, phyllopyrrol, isohemopyrrol (designated hemopyrrol by Fischer) and cryptopyrrol,

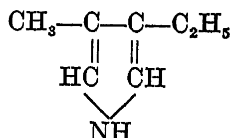


Isohemopyrrol

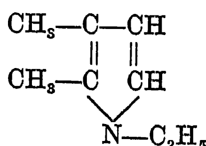


Cryptopyrrol

of which isohemopyrrol is most abundant, are the chief, but not the only, constituents of the mixture. O. Piloty and J. Stock[16] fractionated considerable quantities of the hemopyrrol mixture by Willstätter and Asahina's method and isolated from it the following additional constituents:



Hemopyrrol *a*, that is, 3, 4-methylethylpyrrol.



Hemopyrrol *e*, that is, 1-ethyl-2-3-dimethylpyrrol,

which is obtained only in the form of a bimeric derivative.[17]

Willstätter and Asahina's hemopyrrol (the picrate of which melts at 108–109°) is considered by Piloty and Stock as a mixture of iso-

[14] Ber. d. d. Chem. Ges. 44: 2758. 1911: 45: 2526. See R. Willstätter and Y. Asahina. Ber. d. d. Chem. Ges. 44: 3707. 1911.

[15] Ber. d. d. Chem. Ges. 45: 1979. 1912.

[16] Ann. d. Chem. 392: 215. 1912; Ber. d. d. Chem. Ges. 46: 1008. 1913.

[17] According to O. Piloty and K. Wilke the statements regarding hemopyrrol *e* are doubtful (Ber. d. d. Chem. Ges. 46: 1597. 1913).

hemopyrrol, cryptopyrrol and other pyrrols; this interpretation, to be sure, is very uncertain, since Willstätter and Asahina were not at all able in their investigation to resolve the base into components by fractional salt formation with picric acid.

The above displayed formula for phyllopyrrol has been proved to be correct by elegant syntheses carried out by H. Fischer and E. Bartholomäus.[18] Fischer and Bartholomäus, in fact, made the discovery that pyrrols are alkylized by heating with sodium methylate and sodium ethylate. By means of this beautiful method they obtained phyllopyrrol from trimethylpyrrol and from dimethylethylpyrrol. Phyllopyrrol has been prepared synthetically by U. Colacicchi[19] also.

2. Analysis of Hemopyrrol by Means of Fractional Precipitation with Picric Acid.

The example of the fractionation of the hemopyrrol that was prepared from hemin is cited (practically in the words of Willstätter and Asahina) on account of the practical value of the method, even though the homogeneity of one of the three components has become somewhat doubtful.

The cleavage of hemin by the action of hydriodic acid and phosphonium iodide was carried out essentially according to the directions of Nencki and Zaleski[20] and, in fact, with 25–50 g. portions of hemin. Each 25 g. of hemin was first heated with a mixture of 450 cc. of glacial acetic acid and 500 g. of hydriodic acid of specific gravity 1.96 for 1½ hours upon the water bath. The hemin quickly went into solution; the liquor turned reddish brown and then gradually became deep brown as a result of the liberation of iodine. The warming being continued, only 20 g. of phosphonium iodide (instead of the 40–50 according to Nencki and Zaleski) was then introduced in small portions during the course of a half hour. This amount was sufficient to clear the solution so that, at the end, a test sample was clear and bright yellow when water was added to it. The solution was diluted with 1½ times its volume of water and calcined soda was introduced till the reaction was strongly alkaline. The pyrrols can be extracted with ether but they are obtained in a purer state (although with a small loss as a

18 Ber. d. d. Chem. Ges. 45: 466. 1912; Zeitschr. f. Physiol. Chem. 77: 185. 1912.

19 Atti. R. Accad. dei Lincei 21: I, 489 and 653. 1912.

20 Ber. d. d. Chem. Ges. 34: 1002. 1901.

result of resin formation) when steam distillation is carried out. The distilled bases were collected in receivers that contained ether and a dilute caustic soda solution; the extraction with ether was supplemented by salting out.

For further treatment the ether extracts from 300 g. of hemin were united.

In order to separate the hydrogenated pyrrol bases, the whole ethereal solution of the bases is extracted three times by shaking with a 30 per cent monosodium phosphate solution. The strong bases are removed quantitatively without any of the pyrrols accompanying them. The ethereal solution is then washed with a little lye and with water, and dried with sodium sulphate.

Fractional salt formation. The mixture is first resolved by fractional picrate formation into three main fractions which are still impure.

The solution (800 cc.) contained 70 g. of bases, almost 80 per cent of which could be isolated in the form of picrates (155 g.). The calculated amount of picric acid (about 135 g.) is not sufficient, since the picrate of phyllopyrrol, especially, separates well from an excess of ethereal picric acid only. 150–160 g. of picric acid are, therefore, employed and ten fractions of picrate, which may be united into three chief fractions, isolated.

1. Upon the addition of 30 g. of picric acid dissolved in 600 cc. of moist ether, 35 g. of picrate quickly separated. The prisms had a melting point of 116° (not sharp) and a sample, recrystallized from alcohol, had a melting point of 119°.

2. The filtrate from 1, with 10 g. of picric acid in 200 cc. of moist ether, gave, in an hour, 7.0 g. of the same picrate.

3. The filtrate was again mixed with 10 g. of dissolved picric acid and placed in the ice chest for a few hours. It yielded 19.0 g. of picrate (M. P. 116°), consisting of long and short prisms. A recrystallized sample melted at 119°.

These three separations (together with 7a) were united to form the chief fraction, I (about 62 g.); it contained, chiefly, isohemopyrrol.

4. The filtrate produced with 10 g. of dissolved picric acid 6.5 g. more of picrate, needles and small plates with the melting point 108–109°, which did not change on recrystallization.

5. When the same amount of picric acid was added again, a further separation of 12.5 g. of picrate, melting at 109°, was obtained.

6. The filtrate was mixed with double the quantity of picric acid; 11.0 g. of picrate (M. P. 108–109°) separated in 1½ hours.

7. The filtrate was allowed to stand over night in the ice-box after 15 g. of picric acid in 300 cc. of ether had been added; the new precipitate amounted to 9.0 g.; it melted at 108° and consisted of fine needles and stout plates. Plates, mechanically isolated from the mixture, showed the melting point of 118°. It is seen that the separation can not be carried out in a single step. After most of the second base has been precipitated the solution deposits anew some isohemopyrrol salt.

The 7th fraction required a preliminary treatment before it was sufficiently pure to be united with the other crystallizations. It was shaken with a mixture of 1 volume of ether and 2 volumes of ethyl acetate till the undissolved portion consisted of rather pure, stout prisms. The latter were then recrystallized from alcohol and 1.4 g. of long columns (M. P. 115°) were thus obtained: 7a. The alcoholic mother liquor, as well as the ethereal-ethyl acetate solution, together yielded 6.0 g. of picrate (M. P. 108–109°) : 7b.

Crystallizations 4–6 and 7b (36 g.) were united to form the chief fraction, II, and this was treated by further fractionation in order to obtain our second component.

8. 20 g. of picric acid in 400 cc. of ether were added to the filtrate of 7. On standing at 0° no further crystals were obtained. The solution was, therefore, concentrated in a vacuum to 2/3 of its volume and then cooled for 1½ hours with ice. Only 4 g. of picrate (M. P. about 94°) separated as a result.

On examination this preparation proved to be a mixture. It was shaken with 100 cc. of ether for some time and the undissolved portion was separated by filtration. The solution, when brought to a small volume in a vacuum and saturated with picric acid, produced 1 g. of pure phyllopyrrol salt. The undissolved portion was recrystallized from alcohol. At first 1.1 g. of picrate (M. P. 118–119°) separated, then a little that melted at 105° and, finally, when saturated with picric acid, 2.2 g. of phyllopyrrol picrate, mixed with picric acid, separated from the concentrated mother liquor.

From fraction 8, therefore, about 3 g. were obtained for chief fraction, III.

9. The filtrate was concentrated again in a vacuum to half its volume; this time 40.0 g. of picrate (M. P. about 90°) separated as a powder consisting of microscopical prisms. A test sample gave with

caustic soda a base which quickly solidified as a crystalline mass.

10. The mother liquor was saturated with 30 g. of finely powdered picric acid. On standing over night in the ice chest 5 more g. of picrate, with the properties of fraction 9, separated.

Crystallizations 8, 9, and 10, which consisted chiefly of phyllopyrrol salt, furnished the chief fraction, III (about 48 g.), which was subjected to another fractionation.

11. The filtrate from the 10th crystallization, on further concentration in a vacuum, produced only a small quantity of crystalline picrate. The bases were, therefore, liberated and extracted with ether and, after evaporation, the residue was again subjected to steam distillation. There was carried over by this distillation a small quantity of oil, the ethereal solution of which gave with picric acid an additional small separation (M. P. 118°) and a further crystallization (0.5 g.) which melted at about 90°. 15 g. of brown resin remained in the flask after the steam distillation. By warming the resin with hydrogen iodide and phosphonium iodide and with further treatment exactly like that used in the reduction of hemin, pyrrols, or at least a part of the bases that were lost as a result of the resinification, can be again obtained.

Isohemopyrrol.

Pyrrol was isolated from 60 g. of the chief fraction, I, by means of caustic soda and distilled in vacuo; it was then again subjected to fractionation by means of picric acid. This procedure, of course with losses, produced to all appearances a completely homogeneous base.

The crude base was precipitated as picrate with only two-thirds of the theoretically required amount of acid and the precipitate was recrystallized from alcohol. Basic material was recovered from the alcoholic mother liquor and the residual solution from the picrate formation, and picrate was again separated by using half the calculated amount of acid; this picrate was recrystallized just as before. The isomer, admixed with the crude base, remained in the mother liquor.

Isohemopyrrol was finally liberated from the purest picrate thus obtained, and distilled again in vacuo.

Hemopyrrol.

A completely homogeneous preparation was isolated from the chief fraction, II, of the picrates by three additional, systematic applications of fractional salt formation.

Second fractionation. The base liberated from 34.5 g. of picrate was mixed with separate tenths of the amount of picric acid required for saturation.

The first four-tenths gave precipitations (9.8 g.) that had too high a melting point and proved to be mixtures of the picrates that melt at 108 and 119°. After this, there was obtained with the fifth tenth and then, in one operation with the second half of the picric acid, crystallizations (20 g.) of the correct melting point. The base was again liberated from these.

Third fractionation. The picric acid was introduced in still smaller portions this time, namely, in portions of 20 cc. of moist, ethereal solution $\Bumpeq$ 1 g. of acid. The first two precipitates melted at too high a temperature, also the third was still uncertain. The fourth gram of picric acid, on the other hand, yielded 1.1 g. of pure hemopyrrol picrate. 150 cc. of picric acid solution were then introduced at once and, after filtering from 7 g. of picrate of M. P. 180°, another crystallization (3.8 g.), which was just as pure, was obtained by concentration in a vacuum. 1.2 g. more of good picrate were produced from the first three precipitates by special fractionation.

Fourth fractionation. The pyrrol was again liberated from the 13.1 g. of picrate. The precipitate (0.4 g. of M. P. 107–108°) that was formed by the first tenth of the required picric acid was thrown away for safety's sake. The subsequent precipitations, 11.2 g. in all, of picrate of melting point 108° formed our initial material for the preparation of free hemopyrrol.

The distilled base, in fact, again produced picrate of melting point 108° and, on repeated recrystallization, no fraction that melted at a different temperature could be observed.

Phyllopyrrol.

The base was freed from 48 g. of the most soluble picrate (chief fraction, III) and an attempt was made to separate admixed hemopyrrol by the addition of picric acid in small portions to the ethereal solution (300 cc.). Only a little resin was precipitated by the addition of 66 cc., and then of 50 cc., of picric acid solution (containing 5 g. in 100 cc.) and concentration to a third of the volume; on the addition of 50 cc. more of picric acid only 0.5 g. of crystals of melting point 92° precipitated; these were thrown away. The filtrate, when saturated

with 25 g. of finely powdered picric acid, produced 30 g. of crystals and, when moderately concentrated, 3 g. more of picrate of indefinite melting point 92°. The phyllopyrrol was liberated from this fraction of 33 g.

3. Isolation of the Hemopyrrols from Chlorophyll.

A mixture of phytochlorin and phytorhodin, such as is obtained in the hydrolysis of good pheophytin preparations, was subjected to reduction in a glacial acetic-hydrochloric acid solution with granulated tin at 100°. In two experiments, each with 50 g. of material, the ethereal solution of the volatile bases, after purification with phosphoric acid, gave immediately with picric acid a precipitate (Expt. I, 4.7 g.; Expt. II, 5.5 g.) with a melting point of about 110° which rose to 116° when the precipitate was recrystallized from alcohol. After the filtrate had been strongly concentrated the salt, of melting point 92–93°, of the crystallizing base (Expt. I, 4.1 g.; Expt. II, 2.5 g.) could also be separated by the use of pulverulent picric acid.

With the use of the same method 10 g. of very pure phytochlorin *e* produced a picrate fraction which fused at 117°, and a second one that melted at 108° (together, 2.95 g.); phyllopyrrol remained in the mother liquor.

A similar yield was obtained upon reduction cleavage by Nencki and Zaleski's method. The manner of treatment was the same as with hemin. 50 g. of a chlorin-rhodin mixture were dissolved in 1 l. of glacial acetic acid and 1 kg. of hydriodic acid of specific gravity 1.96, heated 1 hour at 100°, and then phosphonium iodide was added till the solution became clear brown.

The ethereal solution of the bases that are volatile, when steam distilled, produced

1. 7.7 g. of picrate, M. P. 116°
2. 1.7 g. " " " 113°
3. 6.8 g. " " " 94°

The first two precipitates were mixtures of the two hemopyrrol picrates. The last crystallization consisted of rather pure phyllopyrrol. This base, which was liberated from two similar picrate preparations, distilled at 89° under 10 mm. pressure, and melted at 55–57° after being pressed on a porous clay disc.

A similar reduction experiment was carried out with 25 g. of pure phytochlorin *e*. The first picrate precipitate (3.8 g.), after recrystal-

lization from alcohol, had a melting point of 118–119°; the second (1.5 g.) had a melting point of 108°. Phyllopyrrol was not isolated in this case.

In these experiments with phytochlorin, the yield of both hemopyrrols together amounted to 10 per cent, and the yield of phyllopyrrol to 5½ per cent, of the weight of the phytochlorin. Up to this time there had been isolated, of all the bases, roundly only ¾ths of the theoretical amount; 1 molecule of base from 1 molecule of phytochlorin.

The following yields of hemopyrrols were obtained with other chlorophyll derivatives in preliminary determinations. They were simply determined in the form of the mercuric chloride compound by Nencki and Zaleski's method; *i.e.*, weighed in a Gooch crucible.

1 g. of ethyl chlorophyllide (*a* with *b*) produced 0.923 g. of mercury compound; that is, 0.15 g. of hemopyrrols.

1 g. of phylloporphyrin produced 2.04 g. of mercury compound, which is 0.33 g. of hemopyrrols.

2.5 g. of phylloporphyrin produced 5.12 g. of mercury compound, which is 0.82 g. of hemopyrrols.

One molecule of phylloporphyrin, therefore, produced almost 1.4 molecules of hemopyrrols precipitable by mercuric chloride.

4. Description of the Pyrrols from Chlorophyll.

Phyllopyrrol, $C_9H_{15}N$

distills constantly, under 10 mm. pressure, between 88–90° and boils at 213° with the barometer at 725 mm. (determined by Schleiermacher's method). Phyllopyrrol crystallizes from ether or petroleum ether in small, snow-white, irregularly defined, rectangular, mica-like, lustrous plates of melting point 66–67°. It is very unstable when exposed to the air.

Potassium reacts with a turbulent evolution of hydrogen and yields a crystalline potassium salt. The base dissolves very slowly in 1 per cent hydrochloric acid; more quickly in more concentrated mineral acids.

Phyllopyrrol does not exhibit the familiar color reactions of the pyrrols (distinguishing it from the other hemopyrrol components) and its aqueous solution (containing acetic acid and some alcohol) is not precipitated by mercuric chloride.

The picrate crystallizes in small, dark yellow prisms of melting point 95°.

The tetrahydro-compound is always obtained, mixed with pyrrolin, when the phyllopyrrol is heated with hydrogen iodide and phosphorus to 250°, but the hydrogenation can be carried to completion with hydrogen and platinum according to the well-tested procedure of Willstätter and Waser[21] for unsaturated bases. Pyrrolidin distills between 160 and 164°; it is a piperidin-like smelling, easily mobile fluid of $d_4^0 = 0.843$.

Isohemopyrrol, $C_8H_{13}N$

boils at 198° under 725 mm. pressure, and at 88° under 11–12 mm.; it is a colorless (in contrast with older data on hemopyrrol), non-fluorescent oil with a clinging smell; $d_4^{20} = 0.915$. It solidifies easily to a laminated, crystalline mass of melting point 16–17°.

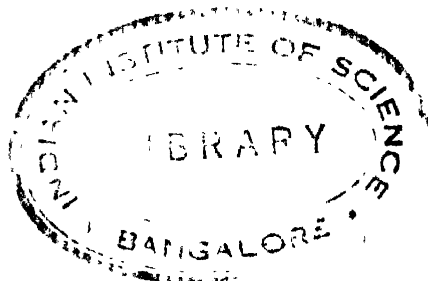
The styphnic acid salts are especially suitable for the identification of the pyrrols since they are distinguished by their power of crystallization, difficult solubility and their stability. The styphnate of isohemopyrrol forms yellow, rectangular prisms (M. P. 136°), which are difficultly soluble in cold alcohol.

The tetrahydro-derivative boils about 43° lower than does the pyrrol and is much lighter than it; $d_4^0 = 0.845$.

Hemopyrrol.

The pyrrol from the chief fraction, II, of the picrates also corresponded to the composition, $C_8H_{13}N$; it distilled at 86–87° under 12 mm. pressure and did not crystallize. The melting point of the styphnate lay at 120–121°. Oxidation with nitrous acid by Piloty's method produced a methylethylmaleicimid oxime, melting sharply at 201°, while there was formed from isohemopyrrol, under the same conditions, an isomer whose melting point was 219°.

[21] Ber. d. d. Chem. Ges. 43: 1176. 1910.



XXIII. THE CARBOXYL-FREE PARENT SUBSTANCES: ETIOPHYLLIN AND ETIOPORPHYRIN.[1]

1. Preparation.

Decarboxylation by heating with methyl alcoholic potash in a closed vessel led smoothly to the monocarboxylic acids, but not to products beyond these. Decomposition took place at about 250° with the formation of amorphous, brown products and hemopyrrols, so that the carboxyl-free phyllin or porphyrin could not be formed by using this method. The duration of heating in this experiment was probably too long. Phylloporphyrin remained unchanged when boiled with quinoline and acridine; decarboxylating bacteria (we thank the firm of F. Hoffman-La Roche in Grenzach for a pure culture of bacteria that decarboxylized histidine well) did not attack rhodophyllin.

Decarboxylation is most successfully carried out by heating small amounts of the phyllins and porphyrins in a test tube with soda lime for a short time; it is necessary, however, to find a suitable temperature at which the carbonic acid is just split off without destroying the sensitive material. The dissociation temperature of the alkali salts that are used is undoubtedly higher than the temperature at which the product of the reaction decomposes during the period of the experiment.

Carbon dioxide is split off from the phyllins with noticeably greater ease than from the porphyrins; less by-product is formed and almost no hemopyrrol. It is easier, therefore, to prepare etiophyllin than etioporphyrin, and the best method for the preparation of carboxyl-free porphyrin is by way of the phyllin, which is freed from magnesium by means of rather concentrated acid. Conversely, the magnesium can be again introduced into etioporphyrin by Willstätter and Forsen's method; namely, by the action of Grignard's reagent.

In the preparation of the phyllin special care is to be observed that no foreign metal (iron, for example) takes the place of magnesium;

[1] Paper XXIII.

the porphyrin can be entirely freed from foreign metals, but phyllin cannot.

Carboxyl-free compounds are, in general, difficult to purify. Because of their indifference toward alkalies, the acid admixtures can be removed. Furthermore, etiophyllin, in ethereal solution, can be washed with dilute hydrochloric acid (toward which it shows great stability) and the porphyrin fractionated by means of it according to the general method for the separation of chlorophyll derivatives. But these methods were not sufficient. An unstable, magnesium-containing substance stubbornly remained admixed with the etiophyllin, and could only be separated from the ethereal solution by precipitation with petroleum ether. The purest etioporphyrin was isolated from its crystalline styphnate.

Naturally, a high yield is not to be expected when carbon dioxide is split off by means of soda lime, because a portion always remains unchanged and another portion is destroyed. Nevertheless, 14 per cent of the theoretical yield of etiophyllin is obtained from rhodophyllin and a yield of about 10 per cent of etioporphyrin from phylloporphyrin.

The same etioporphyrin was obtained from phyllo- and from pyrroporphyrin and it is identical with that obtained from rhodophyllin by way of etiophyllin.

Etiophyllin. Potassium rhodophyllin is carefully triturated with 4–5 times as much pure (iron-free) soda lime. Portions containing about 0.05–0.1 g. of the potassium salt are carefully, but quickly, heated in test tubes over a small flame, constant motion preventing the substance from adhering to the glass wall. On equable heating a sudden change of color—from light gray to brown—is observed; at the same time the odor of hemopyrrol becomes noticeable. At this point heating should be interrupted immediately and the temperature quickly lowered by cooling in a dish with powdered copper. The cooled reaction product is moistened with a little water and extracted by pure (fat-free) ether with brief warming and shaking. The solutions obtained from 10 g. of potassium rhodophyllin were united and the whole purified by the successive action of alkali and acid. Since etiophyllin does not show any pronounced acid properties, the ethereal solution is first thoroughly shaken with pure (zinc and copper-free), concentrated methyl alcoholic potash. The lye takes up acid by-products and a portion of the etiophyllin, and turns brown. Without separating it from the ether, the alkaline layer is diluted with water, whereupon

amorphous floccules separate and all the phyllin reenters the ether. The solution is tested for etioporphyrin by shaking it with 4 per cent hydrochloric acid. The phyllin is stable toward this acid; admixed porphyrin dissolves in it with a reddish color. No porphyrin is formed, however, when the procedure has been perfect. Still, it is recommended that the ethereal solution be vigorously shaken with a few hundred cubic centimeters of 5 per cent hydrochloric acid about ten times, as a result of which brown floccules are again precipitated. Finally, all the acid is removed by means of dilute ammonia. The solution, when evaporated to 2 cc., congealed in one experiment to a crystalline paste; the preparation could be recrystallized from ether, in which it is very easily soluble. The solution, without drying, was filtered and concentrated to 1 cc. The etiophyllin then separated in beautiful, bluish violet, crystalline aggregates which showed tabular and prismatic forms under the microscope and were rose red to violet, depending upon their thickness, in transmitted light. The yield of crude product amounted to 0.5 to 0.6 g.; of recrystallized substance, 0.4 g.

In other experiments etiophyllin did not crystallize, probably because it was less pure. Its originally bluish tinted, red solution in ether, or in benzol, also turned off-color on long standing. An admixture, which fluoresced the same as the phyllins but had an ugly brown color, could be precipitated with petroleum ether after concentration. When treated with acid it gave a porphyrin, which was brownish yellow in ethereal solution, along with an abundant formation of amorphous floccules. The by-product, itself unstable, appears to impair the stability of the etiophyllin. It has no acid properties but contains oxygen; perhaps it did not take up oxygen until subjected to the operations. The etiophyllin after its separation is pure and quite stable, even in dilute, ethereal solution. This has a very beautiful, fuchsin red color.

Formation from Etioporphyrin. The concentrated solution in dry ether is completely precipitated as a bright red precipitate by means of magnesiummethyl iodide; the strongly fluorescent phyllin solution is formed when the suspension is shaken with primary phosphate.

Etioporphyrin. The purified solution of etiophyllin (from 6 g. of potassium rhodophyllin) is shaken thoroughly with 20 per cent hydrochloric acid; decomposition takes place at once and the porphyrin enters the acid with a violet red color. It is transferred from this into fresh ether by neutralization with ammonia and again extracted by 4

per cent hydrochloric acid. To do this, repeated extractions are neces sary. The ether retains a feebly brownish color only. By approxi mate neutralization the porphyrin is again brought into ether and con centrated to 10–20 cc.; the porphyrin now begins to separate an(forms a beautiful, violet lustered, crystalline crust (0.6 g.).

In order to prepare etioporphyrin from phyllo- (and pyrro- o: rhodo-) porphyrin the intimately triturated mixture with soda lime i: heated, just as briefly as (but to a higher temperature than) in th(case of the phyllin, till a sudden, violent evolution of vapor occurs After moistening and extracting with ether, the combined solution: (from numerous small portions) were washed four times with 1 pe] cent hydrochloric acid. In this way a by-product, which occurs ir small amounts, is removed; this enters the acid with a bright red color is strongly basic, and crystallizes beautifully. The ether was ther repeatedly shaken with 10 per cent ammonia and, by this means, th(ammonium salt of an acid was precipitated in floccules; this acid i: more weakly basic than are phyllo- and pyrroporphyrin. If the etio· porphyrin is isolated at this point it will still contain mineral constitu· ents; it is, therefore, transferred at least once from the ether into 1(per cent hydrochloric acid. A little metal-containing product remain: in the ether when this is done. The etioporphyrin solution is then simply diluted and extracted with ether again. The substance, which is difficultly soluble when in a pure state, is permitted to crystallize from the moderately concentrated ethereal solution; it forms crystal· line aggregates. The yield amounts to 6–10 per cent of the initial material.

2. Description.

The composition of etiophyllin corresponds to the formula $C_{31}H_{34}N_4Mg$; it yields almost 8 per cent ash which consists of pure MgO.

Its ethereal solution is stable towards 4–7 per cent hydrochloric acid when shaken with this and allowed to stand for several hours; it is, therefore, much more stable toward this acid than are the carboxylic acids of the phyllin series.

Only when the concentration of the hydrochloric acid reaches 15 per cent is a small portion immediately extracted by it, although the ethereal solution still remains unchanged in its color and fluorescence. Likewise, when its ethereal solution is mixed with glacial acetic acid etiophyllin does not easily lose its magnesium. The fluorescence dis- appears only after the mixture has stood for some time.

The behavior of a petroleum ether solution of etiophyllin towards dilute acid is entirely different and very striking. The color changes immediately to the bronze hue of etioporphyrin, even when the strength of the hydrochloric acid is but 0.05 per cent; with more dilute acid than this, however, there is no change. Etiophyllin in petroleum ether behaves towards 1–3 per cent hydrochloric acid exactly the same as does completely purified etioporphyrin. A portion of the porphyrin goes into solution, another portion crystallizes as hydrochloride in long, glittering needles.

The substance goes into solution with a red color when it is warmed with methyl alcoholic potash; it precipitates if the alcohol is boiled off and, on dilution, dissolves in ether, unchanged.

Etiophyllin dissolves in ether and in alcohol with extreme ease and it is also very easily soluble in the other organic solvents, with the exception of petroleum ether in which it is difficultly soluble. The alcoholic solution is bluish red in color and exhibits marked fluorescence as does the ethereal solution also; when diluted it shows a beautiful, violet red color.

On evaporation of its petroleum ether solution the phyllin crystallizes in rhombic plates which are similar to carotin but deeper red. These rhombs are often rounded, like spindles, and are inclined to form twins and penetration triplets.

Its solubility does not change when it is dried in a high vacuum. In a melting point tube the substance sinters above 160°; it gradually melts at about 205° and is still unaltered at 250°.

Etioporphyrin, $C_{31}H_{36}N_4$. The hydrochloric acid number of the porphyrin is 3; its distribution number for 3 per cent hydrochloric acid is 40. Its melting point is approximately 280°; the powder has a color similar to that given by alizarin with a chrome mordant. The porphyrin is slightly soluble in cold alcohol; it is much more, though still rather difficultly, soluble in boiling alcohol. The alcoholic solution is brownish red, with a somewhat more reddish tint than that of the ethereal solution, and it fluoresces considerably less than the phyllins, though more strongly than pyrroporphyrin. It is easily soluble in acetone and considerably so in warm glacial acetic acid. It dissolves easily in formic acid with a beautiful, bluish tinted, red color. The substance remains unchanged when boiled with methyl alcoholic potash.

Etioporphyrin forms characteristic complex compounds with salts of the heavy metals. Its glacial acetic acid solution turns pure red in

color when warmed with zinc acetate; with copper acetate it acquires a red violet color; this is likewise red when transferred to ether. The copper derivative resists the action of concentrated hydrochloric acid; the zinc compound is hydrolyzed by dilute mineral acids in the same way as is etiophyllin.

Etioporphyrin forms beautiful salts with picric acid, chloroplatinic acid and other acids. The picrate is precipitated in red floccules from ether; when it separates slowly it forms beautiful red prisms with domed extremities. The styphnate (M. P. 170°) crystallizes in rose-colored prisms with frequent twin formations. A reddish violet crystalline precipitate is obtained with ethereal gold chloride.

The hydrochloride crystallizes in long olive brown needles when the ethereal porphyrin solution is slowly mixed with ether that contains hydrochloric acid.

The hydrochloric acid solution of etioporphyrin does not react with dimethylaminobenzaldehyde.

Absorption Spectra.

Etiophyllin. The absorption spectrum (Fig. 16) is very similar to that of pyrrophyllin, especially of recrystallized pyrrophyllin. In addition to the characteristic absorption bands in the green and yellow regions there appears a less intensive, characteristically ribbed band in the blue. In addition to three weak bands in the red the spectrum shows, in the visible region, the two chief bands in the yellow and the green, a feebler band where the green passes into the blue, and the divided band which lies in the blue and indigo blue.

0.0488 G. IN 1 L. OF ETHER (0.001 MOL. IN 10 L.).

Thickness of layer, in mm.	10	20	40
Band I	—	646 | 640	646 . 640 | 636
" II	—	622 | 619	622 | 619
" III	—	614 | 610	614 | 610
" IV	583—572	585—572	586—570 |
" V	550 | 548—538 | 532	559 . 550—531 . 527	560 . 555—526 . 523
" VI	504 | 491	507 . 487	508 . . . 486
" VII	—	471 | 458 . 454 | 447 . . 445 |	472 . 467 | 460 . . 455 | 449 . . . 445 |
End absorption	422—	430—	434—

The sequence, arranged according to the intensity, is: end absorption, V, IV, VI, VII, I, II, III.

Etioporphyrin. The spectrum is brightened in the yellow and green regions as a result of the removal of the magnesium, while the absorption is increased in the blue region. Etioporphyrin shows a complicated spectrum (Fig. 16), similar to that of pyrroporphyrin, with chiefly four strong bands. One of these lies in the orange, a characteristically divided one lies in the yellow green region, the next is in the green and the fourth, which is the strongest and particularly distinct (VIII), lies in the blue. In addition, there is also a significant absorption in the violet.

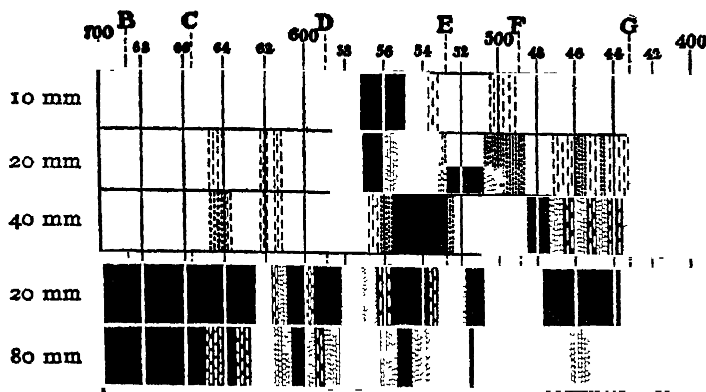


FIG. 16. Absorption spectra of etiophyllin (above) and of etioporphyrin (below).

0.0464 G. IN 1 L. OF ETHER (0.001 MOL. IN 10 L.).

Thickness of layer, in mm.	10	20	80
Band I	—	—	649 . 643
" II	624——620	624—619 |	633 | 626—618 ...
" III	612 | 609	613 . 609	609
" IV	598 | 593	598 | 593	598 .. 593 |
" V	580 . 572	581 ... 572	588 . 582—563 ..
" VI	568 ... 565 | 556	569——564 | 556	556
" VII	533 | 530 .. 525 ... 523 . 518	536 | 531——522 .. 518	545 . 538—519
" VIII	505—479	507—479	514—475 ... 463 .
End absorption	427—	435—	452—

The sequence, arranged according to the intensity, is: end absorption, VIII, VII, II, VI, V, III, IV, I.

XXIV. SYSTEMATIC DECOMPOSITION OF HEMIN.

1. Preparation of Hemin.

Improvement of the Method of Schalfejeff.

In Schalfejeff's method, as well as in that of Nencki and Zaleski, fresh defibrinated blood is treated with hot glacial acetic acid which contains sodium chloride. According to W. Küster (Abderhaldens' Handbuch der biochemische Arbeitsmethoden, Vol. II, p. 619) at least four times as much glacial acetic acid as blood is required.

But this can be reduced to three times that of the blood and, by the use of a modified method, significant quantities of hemin can be successfully obtained by the simplest means.[1]

Five round four l. flasks, each charged with 3 l. of glacial acetic acid to which there has been added a little solid table salt or 1 cc. of saturated sodium chloride solution per liter of glacial acetic acid, are heated upon a steam bath or over gas burners.

Into each flask, whose contents are kept in motion by frequent rotation or by means of an effective stirrer, defibrinated blood (filtered through filtering cloth) is allowed to flow in a thin stream from a dropping funnel, while continuously heating so that the temperature of the contents of the flask does not fall below 95°.

The tip of the discharge tube of the dropping funnel lies midway between the stirrer and the wall of the neck of the flask at such a height that the glacial acetic acid vapor does not rinse it and plug it with protein coagulum. Contact between the in-flowing blood and the walls of the flask should be avoided. After the introduction of the blood the liquor is kept slowly boiling for a quarter of an hour whereupon a quite large portion of the hemin separates in glittering crystals.

After 2–3 days the somewhat viscous mother liquor, from which only a little hemin crystallizes on longer standing, is decanted carefully from the crystalline paste and this is filtered upon a suction filter

[1] Revised from Ann. d. Chem. 373: 232. 1910 and 385: 197. 1911.

through several layers of filter cloth. The crystals are washed with dilute acetic acid, water, alcohol, and ether.

The yield, probably fluctuating with the composition of the blood, amounts to 4½ to 5½ g. of pure hemin per liter of blood.

Preparation from Centrifuged Blood.[2]

We centrifuge undiluted, defibrinated blood, although it is usually customary to dilute it considerably with sodium chloride solution before centrifuging.

By the use of a laboratory centrifuge manufactured by Gebrüder Heine in Viersen and which has a rim diameter of 66 cm., makes 3,000 r.p.m., and holds 1.9 l. in the 6 glass vessels, half of the cow's blood is deposited as blood corpuscle paste. It is difficult, however, to remove the serum completely, so that 1,100 cc. of blood corpuscles are obtained in practice from 1,900 cc. of blood. The volume of the concentrated blood remains unchanged when it is mixed with a 0.9 per cent sodium chloride solution and recentrifuged.

A high-speed Jouan centrifuge—model C, electrically driven, running 9,000 r.p.m., with containers of 120 cc. content, with outer diameter of 24 cm.—made by Leune of Paris, works far better, and especially so when its so-called "bol metallique" (inner diameter 18 cm., vol. 1½ l. and 5,000–5,500 r.p.m.), which permits the centrifuge to be filled and emptied while in motion, is used. In spite of the smaller path traversed per second the service rendered by this machine is better than that of the large centrifuge.

From a liter of cow's blood, 635–640 cc. of clear serum (in another test, 660 cc.) are drawn off after 10 minutes, that is, approximately the largest possible amount. If the centrifuge is then stopped the bowl contains the suspension of unchanged blood corpuscles (340–365 cc.). But if the revolving centrifuge is emptied by means of the brass tube of the apparatus, the blood corpuscles are crushed by their impact against the wall of the discharge tube and a laky, beautiful deep red, oxyhemoglobin solution runs from the bowl; this is all the oxyhemoglobin of the blood, mixed in a clear solution with an exceedingly small amount of water. Under the microscope only the outlines of the blood corpuscles are recognizable in the red solution.

When the undamaged blood corpuscle paste, as obtained by slowly stopping the centrifuge, is washed with a sodium chloride solution and

[2] Unpublished.

centrifuged in the bowl its volume does not decrease in the glasses of the centrifuge, even at 9,000 r.p.m.; washing is, therefore, of no value in the preparation of hemin. After the blood corpuscles are crushed they can, of course, not be washed any more, since the oxyhemoglobin is miscible with the sodium chloride solution. The use of the oxyhemoglobin solution for the preparation of hemin has the advantage over the elaboration of the blood corpuscle paste that the apparatus is more easily emptied and that larger quantities may be run in continuously and removed without stopping the centrifuge. The centrifuging of the blood and the treatment of the resultant hemoglobin solution with glacial acetic acid can be conveniently carried out at the same time. The process is just as simple and the hemin is just as pure as that obtained by the older methods. Instead of using three or four times as much glacial acetic acid as blood it is sufficient to employ equal amounts of glacial acetic acid and blood.

Method of Procedure. Fresh, defibrinated cow's blood, in liter portions, is permitted to flow through a 2 mm. wide tube into the "bol," rotating at 3,000 r.p.m. The number of revolutions is increased in a minute to about 5,500 and the centrifuge allowed to run for 10 minutes at this speed. The almost colorless serum is now drawn off by slowly lowering the discharge tube till the liquor that runs off suddenly turns deep red in color and the laky oxyhemoglobin solution is then run into another vessel till a slight hiss in the centrifuge betrays the contact of the tube with the walls of the "bol." The "bol" is then emptied to within a few cubic centimeters and the discharge tube screwed back. Sufficient resistance is now again introduced in the electromotor line to cause the "bol" to rotate at only 3,000 r.p.m. and it is recharged.

In each of several 5 l. round flasks with wide necks 2 l. of glacial acetic acid, to which 10 g. of sodium chloride has been added, are heated to boiling over a gas flame and the oxyhemoglobin solution (0.7 l.) from 2 l. of blood is allowed to drop during the course of a half hour into the gently boiling solution which is rapidly agitated by a stirrer. Contact of the oxyhemoglobin solution with the walls of the flask and with the stirrer must be avoided during this process.

The deep brown fluid is then kept gently boiling 10 more minutes and a liter of distilled water is then run in during the course of a quarter of an hour. Most of the hemin then separates in large, lustrous crystals; when working more rapidly it crystallized in such a fine state that the filtration required for its isolation became very difficult.

The crystallization becomes complete on standing a day. The crystals are then filtered through double filtering cloth from the mother liquor, which is only slightly colored, and washed to a slight extent with acetic acid, water, alcohol and ether. The preparation is so pure that no recrystallization is required.

The hemin yield amounted to 4.6–5.2 g. per l. for cow blood which had given 4.2 g. of hemin when worked up according to the older method described above.

2. Hematoporphyrin.[3]

Hemin cannot be as easily freed from iron as chlorophyll is from magnesium. The formation of hematoporphyrin is a more complex reaction. In an effort to explain this reaction attempts have often been made, though these have till now been in vain, to isolate intermediate products of the formation of hematoporphyrin by means of hydrobromic acid. Willstätter and Fischer have now found in this reaction, when the iron is split off with hydrobromic acid in an aqueous or glacial acetic acid solution or where there is no solvent, a series of intermediate products which contain bromine.

On treatment with concentrated aqueous hydrobromic acid (sp. gr. 1.78), a transformation of the hemin powder, which remains mostly undissolved, is observed in a day's time. New, lustrous crystals, obliquely cut prisms, which are very similar to hemin but which contain 2 molecules of added hydrobromic acid, are formed. They correspond to the formula,



$$C_{33}H_{34}O_4N_4FeBr_3.$$

This bromide is distinguishable from hemin by being easily soluble in alcohol with an intensive reddish brown color and also by being appreciably soluble in moist ether. It dissolves easily in concentrated sulphuric acid with a bluish tinted, red color, while hemin slowly gives a greenish red solution. The new compound still contains the iron firmly bound, but it splits off the hydrobromic acid easily; for example, on heating in a high vacuum; hemin is reformed by this.

A second intermediate product, which likewise still contains iron but which is richer in bromine, of the formula,



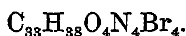
$$C_{33}H_{35}O_4N_4FeBr_4,$$

is obtained with glacial acetic-hydrobromic acid; this is best done with acid of a concentration not suitable for solution. For example, hemin

[3] Paper XXIII.

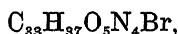
is transformed by the action of a somewhat too weak glacial acetic-hydrobromic acid (sp. gr. 1.40) into this bromide. It is, likewise, easily soluble in alcohol but not in ether.

A third intermediate product can be precipitated by means of dry ether as a clear red powder from the solution of hemin in glacial acetic-hydrobromic acid. It is already free from iron and corresponds to the formula,



$$C_{33}H_{38}O_4N_4Br_4.$$

Finally there appears in the form of its acetyl derivative a carboxylic acid with one atom of bromine and of the composition,



$$C_{33}H_{37}O_5N_4Br,$$

when the glacial acetic-hydrobromic acid solution is first saturated with solid sodium acetate and then diluted instead of being mixed with water, allowed to stand, and then neutralized for the preparation of the porphyrin. This bromine-containing acid crystallizes from its ethereal solution in beautiful pyramids; it is easily hydrolyzed to hematoporphyrin.

The most important iron-free intermediate product of hematoporphyrin formation is formed by splitting the iron off by means of liquid hydrobromic acid; it is a bromide of the formula,



$$C_{33}H_{39}O_4N_4Br_5.$$

On careful neutralization with dry sodium carbonate it produces an ether-soluble acid which contains two bromine atoms. It is easily hydrolyzed to pure hematoporphyrin by means of hydrochloric acid and on standing with methyl alcohol it forms a beautifully crystallizing dimethylether-dimethylester of hematoporphyrin.

It may be concluded from the observed intermediate products that two molecules of hydrobromic acid are first added to hemin and that the complex linkage of the iron is loosened by this; the bromine atoms are then replaced by hydroxyls in the formation of porphyrin.

Hematoporphyrin has heretofore been known exclusively in the amorphous state. The hydrochloride alone was obtained in the crystalline form by Nencki and Zaleski[4] and by later experimenters.[5]

[4] Gesammelte Arbeiten von Nencki. II, pp. 77 and 754.

[5] W. Küster in Abderhalden's Handbuch der biochem. Arbeitsmethoden II. 623. 1910; H. Fischer, E. Bartholomäus and H. Röse. Zeitschr. f. physiol. Chem. 84: 262 and 282. 1913.

Hematoporphyrin itself is obtained in a homogeneous, crystalline state by means of the following method.

It is important for the preparation of hematoporphyrin to use glacial acetic-hydrobromic acid of a very definite, appropriate concentration, namely, of specific gravity 1.41 (determined at 0°). In this way the time given by Nencki and Zaleski is considerably shortened and complete solution of the hemin is attained. Neither a stronger nor a weaker acid is found to be suitable for solution of the hemin and elimination of the iron. For example, the acid of sp. gr. 1.38 sold by Kahlbaum is not very serviceable and even an acid of sp. gr. 1.40 is not suitable.

The unpulverized hemin is introduced into ice cold hydrobromic acid contained in a well-stoppered flask. For example, 10 g. portions are introduced into 250 cc. of acid of specific gravity 1.41 and shaken at room temperature for a day. The hemin forms a clear solution in this time. The liquor is poured into 3 l. of water and is freed from the very few undissolved particles by filtration. The solution is now permitted to stand 3 hours for the hydrolysis of the bromine compound that has been formed and the hematoporphyrin is then precipitated with concentrated sodium acetate solution. Iron is contained in the crude product; otherwise it is a homogeneous substance.

Hematoporphyrin is best identified by its hydrochloric acid number; if a good crude product is separated into fractions, these agree in their distribution between hydrochloric acid and ether.

In order to obtain the porphyrin free from iron it may be dissolved, according to Nencki, in dilute caustic soda and reprecipitated with acetic acid. The substance is also freed from mineral constituents upon its conversion into the crystalline state.

The flocculent precipitate of hematoporphyrin is filtered upon a filtering cloth and washed with water. The still moist preparation, advantageously one that has been reprecipitated from a caustic soda solution, is now dissolved in 1 l. of alcohol and the solution introduced into 25 l. of ether, distributed among five 7-liter separatory funnels. The alcohol is then washed out by allowing tap-water to flow for about 20 minutes through each separatory funnel. When this is done a certain quantity of a flocculent calcium salt is precipitated, which is collected in order that it may be decomposed with acid and the liberated porphyrin returned to the main portion. The ethereal solution is dried with sodium sulphate and concentrated to 1 l. When

this volume is reached the hematoporphyrin begins to precipitate, even when warm. The ethereal solution is now allowed to stand at room temperature and the porphyrin is obtained as a glittering, violet crystallization, which consists of beautifully rounded, rectangular plates, which are reddish brown by transmitted light. The yield of the portion that crystallized was 4.5 g.

The crystalline hematoporphyrin, when dried in a desiccator, has a composition corresponding to the formula, $C_{33}H_{38}O_6N_4$. It loses a molecule of water at 105° under 0.03 mm. pressure and a second molecule under the same conditions upon the introduction of a receiver cooled with liquid air.

Traces of hematoporphyrin are extracted from its ethereal solution by 0.03 per cent hydrochloric acid. It is abundantly extracted (about 2/3) by 0.1–0.15 per cent acid and almost completely by 0.4 per cent acid.

3. Cleavage of Iron from Hemin by Liquid Hydrobromic Acid.[6]

As a result of the action of hydrobromic acid upon hemin in a closed tube at room temperature the bromide, $C_{33}H_{39}O_4N_4Br_5$, is obtained; that is, the hydrobromic acid salt of a bromine-containing carboxylic acid. This salt may be separated from iron bromide. The carboxylic acid, which contains two bromide atoms, is liberated from the salt.

5 g. of hemin crystals are placed in a tube which can be sealed and, by cooling with liquid air, about 10 g. of hydrobromic acid gas that has been previously dried with calcium bromide are also condensed in it. The tube through which this is introduced ends about 1 cm. above the Dewar flask in order to avoid its obstruction by the crystallizing hydrobromic acid. After sealing, the tube is allowed to stand a few days. Before opening, the tube is first cooled with carbon dioxide-ether and then with liquid air. The hydrobromic acid is evaporated and the violet, metallic lustered, crude product (10.3 g.), which is deliquescent, is triturated several times with dry ether and in this way freed from ferric bromide. The bromine compound is more easily obtained free from iron by repeated solution of the crude product in acetone and precipitation with ether. The pentabromide forms lustrous platelets, which are violet to ruby red by transmitted light and which show no crystalline figures.

[6] Paper XXIII.

The hydrobromic acid of this salt must be very carefully neutralized in order to prepare the ether-soluble carboxylic acid of the formula, $C_{33}H_{36}O_4N_4Br_2$. The iron-free pentabromide is dissolved in acetone (1 g. in 20 cc.) and precipitated in a fine state of division by means of 100 cc. of ether. The suspension is then shaken with an excess of anhydrous sodium carbonate till a clear, reddish brown solution is obtained. This is immediately filtered and precipitated with petroleum ether since the bromine-containing acid will not endure the evaporation of its ethereal solution because it is transformed into a hydrobromic acid salt when this is done. After drying, the dark powder (0.6 g.) is easily soluble, with a brownish red color, in alcohol and in acetone but it is no longer soluble in ether.

Hydrolysis of the pentabromide easily produces pure, crystallizable hematoporphyrin; it is only necessary to dissolve the pentabromide in moderately dilute hydrochloric acid in order to obtain immediately the substitution of two bromine atoms by hydroxyls. For example, 5 g. of pentabromide are dissolved with gentle warming in 1 l. of 20 per cent hydrochloric acid and from the filtered solution, after careful neutralization of the acid with ammonia, the porphyrin that has been formed is transferred into considerable ether (about 15 l.). After evaporation to one l., the hematoporphyrin separates as a beautiful, crystalline powder (1.2 g.), in which a typical shape (elongated plates, rounded at both ends) is very often observed.

All the bromine is also separated by the action of methyl alcohol in the cold. The tetramethyl compound that is produced forms large, ruby red pyramids. It is easily soluble in ether, even when dried. Its hydrochloric acid number lies between 3 and 4. It contains only two saponifiable methyl groups. On hydrolysis it produces the dimethyl ether of hematoporphyrin, an acid difficultly soluble in ether, which crystallizes in hemin-like, brownish red plates and is characterized by the hydrochloric acid number, 1.

4. Mesohemin and Hemoporphyrin.[7]

The method of systematic decomposition by heating with alkalies, which had led from chlorophyll through the series of phyllins and porphyrins down to the carboxyl-free parent substances, was applied to hemin and to hematoporphyrin. It was not possible in the case of

[7] Paper XXI.

hemin to split off its carboxyls by the method that was used for the formation of etiophyllin nor could this be done with hematoporphyrin. On the other hand, carboxylic acid cleavage occurs with the porphyrins that are obtained by heating hemin and hematoporphyrin with methyl alcoholic potash.

In the case of the conversion of the blood pigments by means of alkalies at a rather high temperature the same difficulty appeared as with the chlorophyll *b* component. The reaction was not easily accomplished with methyl alcoholic potassium hydroxide alone because the material was partially ruined. On the other hand, beautiful and varied porphyrins are obtained from hemin and from hematoporphyrin when considerable pyridine is present; their formation takes place as a result of a reduction process.

In the case of hemin, the complex linkage of iron remains intact even at 200°. The resultant compound may be freed from iron by means of concentrated sulphuric acid or glacial acetic-hydrobromic acid and furnishes pure mesoporphyrin which checks with M. Nencki and J. Zaleski's[8] description of the reduction product prepared by means of hydrogen iodide and phosphonium iodide. The iron compounds of mesoporphyrin, with the groups, $Cl-Fe=$ and $=Fe(OH)$, are designated mesohemin and mesohematin. The first of these has already been obtained by Zaleski[9] as the result of the action of an iron solution on mesoporphyrin (it is strange that Zaleski used a ferrous salt).

By the same treatment with methyl alcoholic potash and pyridine at 200° a compound similar to mesoporphyrin is obtained from hematoporphyrin; this compound should be called hemoporphyrin. Numerous analyses have given for it the formula,



$$C_{33}H_{36}O_4N_4,$$

while, instead of Zaleski's[10] formula, $C_{34}H_{38}O_4N_4$, the same formula, or the composition,



$$C_{33}H_{38}O_4N_4,$$

is assumed for mesoporphyrin; it probably has two more atoms of hydrogen than hemoporphyrin.

[8] Ber. d. d. Chem. Ges. 34: 997. 1901.

[9] Zeitschr. f. Physiol. Chem. 43: 11. 1904.

[10] Zeitschr. f. Physiol. Chem. 37: 54. 1902.

The two dibasic porphyrins from hemin show a great similarity, as well as some finer differences, which are recorded in the following table:

	Mesoporphyrin	Hemoporphyrin
Hydrochloric acid number	1¼	¾
Distribution number for 0.5% HCl	12	23.5
Behavior toward dilute HCl (5–20%)	first dissolves and then separates at once in coarse floccules	very easily soluble; after long standing only the hydrochloride crystallizes in needles
Behavior toward alcohol	crystallizes well from the hot solution	less completely crystallizable

Mesohemin.

A solution of 3 g. of hemin in 100 cc. of pyridine was carefully stirred in a silver crucible with 50 cc. of methyl alcoholic potash and heated in an autoclave for 4 hours at 200°. The reaction is completed when a sample, after cooling, dissolves in concentrated sulphuric acid with a clear, red color and furnishes a porphyrin whose ethereal solution is not extracted at all by 0.1 per cent acid. If the color in sulphuric acid is still greenish tinted, the alkaline mass still contains a form of hemin; in the case of excessive action by the alkali the sulphuric acid solution is discolored brown.

The alkaline mass is full of glistening, brownish red crystals of potassium mesohematin, long, rectangular prisms that can be isolated by dilution with a little methyl alcohol and washing with ethyl alcohol. They are insoluble in ethyl alcohol but easily soluble in methyl alcohol. Their composition corresponds to the formula,



$$(C_{31}H_{34}N_4)\ (FeOK)\ (COOK)_2.$$

Mesohemin is obtained by acidification with dilute hydrochloric acid and is purified by several fractional precipitations of the crude product from boiling methyl alcohol by means of water. The compound is then transformed into its chloride by solution in boiling glacial acetic acid (2 g. in 50 cc.) and the addition of a little concentrated hydrochloric acid (2 cc.). The dark, precipitated grains (1 g.) may be recrystallized the same as hemin; its solution in pyridine was intro-

duced into warm glacial acetic acid which contained sodium chloride. The mesohemin separated from glacial acetic acid in the well-known forms of hemin crystals while it separated from pyridine-glacial acetic acid in thin plates which were yellowish brown by transmitted light. It is considerably more soluble than is hemin in ordinary solvents; particularly, in acetone it is moderately soluble, while hemin is insoluble therein.

Its composition is $C_{33}H_{36}O_4N_4FeCl$; that is, $C_{31}H_{34}N_4$ (FeCl) $(COOH)_2$ (there is an uncertainty as to the number of hydrogen atoms).

The iron is removed from mesohemin, with the formation of mesoporphyrin, by means of concentrated sulphuric acid, or better, by glacial acetic-hydrobromic acid. Upon comparison, this preparation was found to agree with a preparation made according to Nencki and Zaleski's method.

Hemoporphyrin.

In the treatment of hematoporphyrin with methyl alcoholic potash, about the same method was used as with hemin.

2 g. of hematoporphyrin were heated with 100 cc. of pyridine and 50 cc. of alcoholic potash in an autoclave for 4–5 hours at 200°. Complete transformation is evidenced by the alteration of the basic properties; the ethereal solution of the reaction product no longer colors 0.1 per cent hydrochloric acid.

The silver crucible of the autoclave contained the potassium salt, which had separated in a crystalline form at the bottom; the pyridine could be decanted from the salt. On acidification, the free hemoporphyrin was obtained in an almost quantitative yield, but it was obtained in a pure state only after fractionation with hydrochloric acid by Willstätter and Mieg's method. The crude product was taken up in concentrated hydrochloric acid and transferred to ether by dilution and neutralization. After washing the ethereal solution several times, the hemoporphyrin was extracted from it by means of 2–3 per cent hydrochloric acid. A yellow colored admixture was removed from the acid solution by repeated extractions with ether. Then, after neutralization of the acid the pure porphyrin was extracted from the beautiful red, fluorescent hydrochloric solution by means of ether. On concentration it crystallized in hair-like, reddish brown needles; from a dry ethereal solution it crystallized in thick plates which were red by transmitted light and often showed rhombic outlines.

Hemoporphyrin is insoluble in most solvents but it may be recrystallized from considerable boiling ether. It dissolves easily in hot glacial acetic acid and considerably in the cold acid.

The homogeneity of the hemoporphyrin was proved by decomposing 2 g. of the substance by means of dilute hydrochloric acid into several fractions and comparing these fractions by means of the distribution number.

It is somewhat more strongly basic than mesoporphyrin and its hydrochloride is decidedly more soluble.

5. Etioporphyrin, $C_{31}H_{36}N_4$.

When hemoporphyrin is heated with soda lime it behaves similarly to the isomeric rhodoporphyrin but the cleavage of carbon dioxide proceeds less smoothly so that a large quantity of oxygen-containing by-products appears. Use of the magnesium compound of hemoporphyrin for decarboxylation is preferable since the phyllins lose their carboxyl more easily than the porphyrins.

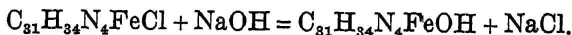
The phyllin of hemoporphyrin is formed directly from hematoporphyrin by heating it in an autoclave at 190° in 6 g. quantities with pyridine (160 cc.), methyl alcoholic potash (90 cc.) and magnesium oxide (2 g.). Its ethereal solution has a fuchsin red color and fluoresces strongly; it is easily converted by acid into hemoporphyrin which has a hydrochloric acid number ¾.

The potassium salt of this phyllin was heated in small portions with soda lime just as was done with the rhodophyllin salt; the etiophyllin was extracted from the alkaline mass with ether; it was impure and, therefore, brownish red. It was necessary to purify the crude product with petroleum ether. If considerable petroleum ether is added to the very concentrated ethereal solution, floccules of an amorphous by-product precipitate immediately and these increase in number on standing. After this treatment the etiophyllin solution is a splendid violet and fluoresces beautifully red. The etioporphyrin formed from this by means of acid (from a petroleum ether phyllin solution even with very dilute acid) was purified by means of its styphnate. The yield then amounted to 0.3 g. from 10 g. of hemoporphyrin.

Etioporphyrin from hemin is difficultly soluble in alcohol and ether; it is easily soluble in glacial acetic acid with a splendid bluish

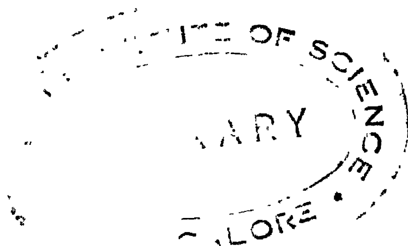
red color. With ether it gives a bronze red solution. The ethereal solution gives up about 2/3 of the substance to 3 per cent hydrochloric acid; the distribution number for hydrochloric acid of this strength is 43. The substance does not react with alkali. It sinters on heating and gradually melts at 265°, or somewhat lower than does the preparation from phylloporphyrin. The melting point of the styphnate is 170°. The etioporphyrin of this preparation agrees, in its properties as well as in its absorption spectrum, exactly with that described in Chapter XXIII. The preparation from hemoporphyrin, however, was probably somewhat less pure, for analysis gave a somewhat too low value for the carbon (79.4–79.6 instead of 80.1 per cent C.).

Iron may be introduced into etioporphyrin when it is warmed in glacial acetic acid with ferric chloride and some sodium acetate; the substitution does not occur without the addition of sodium acetate. The ferric compound, brownish red in ethereal solution, is, as distinguished from etioporphyrin, still indifferent toward 20 per cent hydrochloric acid; stronger acid dissolves the complex compound without cleavage of the iron. Its ethereal solution reacts with alkali at once, turns brown and assumes an intensive yellow tinge. In this way the chloride is transformed into the base according to the equation:



$$C_{31}H_{34}N_4FeCl + NaOH = C_{31}H_{34}N_4FeOH + NaCl.$$

The same reaction can be carried out with mesohemin esters and hemin esters and produces crystallizable hematin esters.



XXV. GRAPHICAL REPRESENTATION OF THE ABSORPTION SPECTRA.[1]

For the measurement of the absorption spectra and their representation in Plates VI and VII, a grating spectroscope was used. This was furnished by Carl Zeiss, of Jena, with a wave-length screw as prescribed by F. Löwe,[2] and contained a grating which gave only a slight dispersion; namely, 3,610 lines to the inch. A slit width of 0.1 mm. was used and an inverted gas lamp was the source of light.

Four different shades were employed for the graphical representation: black for —, oblique hatching for two degrees of absorption, namely — — and . . ., dotted shading for the degrees, . . and ., and line shading for the weakest shadows (|).

The photographs (Plates VIII–XI) were made with a spectrograph that had an Ives grating of great dispersion; namely, 20,000 lines per inch. The apparatus was constructed by Carl Zeiss. When the apparatus was used with an objective of 21 mm. diameter and 420 mm. focal length, the width of the spectrum upon the plate between the wave-lengths 400–700 $\mu\mu$ was 110 mm. and the height 10 mm., so that eight spectra could be taken under one another upon the same plate (9 x 12 cm.).

The valuable experiences that have been published by E. Rost, F. Franz, and R. Heise[3] in regard to the photographing of blood spectra were made use of and our method of procedure was essentially the same as that of these authors. Rost, Franz and Heise produced with a grating of 15,000 lines a spectrum 46 mm. in width; their source of light was a Welsbach lamp.

In this investigation a Nernst lamp had to be used in place of a Welsbach burner. The lamp (a model for projection purposes, 80 candle-power, 220 volts, 0.5 amperes) was fastened upon an optical

[1] See paper XVII.

[2] Verhandl. d. Deutsch. Physikal. Ges. 10: 671. 1908.

[3] Arbeiten aus dem Kaiserl. Gesundheitsamte 32: 223. 1909.

stand together with a condenser lens (of about 8 cm. focal length and 6 cm. aperture), a diaphragm and the absorption trough; all parts were adjustable. The image of the rod-shaped source of light was thrown sharply upon the plane of the slit when the lamp was 11 cm. from the lens and the distance from the lens to the slit was 15 cm. The width of the slit was 0.1 mm. for all the photographs.

In order to determine the position of the lines, the spark spectrum of helium was photographed as the first and last spectrum on each plate. The wave-length scale, printed upon the plates with the Frauenhofer lines and with 4 helium lines, was constructed in 3 sections by measuring the distances between the helium lines, 667.8—587.6—501.6—447.2, and dividing these distances proportionally. The whole scale is, in consequence, not exactly uniform because like angles of refraction, but not like intervals in the plane of the plate, correspond to the differences of wave-length.

The process-panchromatic plates of Wratten and Wainwright, of Croyden, were used.

Although the relatively simple spectra of the derivatives of the blood pigments which were photographed by the Imperial Board of Health show no absorption in the red but bands that begin at $\lambda = 645$ $\mu\mu$, only there is in the case of many chlorophyll derivatives a chief absorption, which begins at $\lambda = 690$ $\mu\mu$, in the red. Since the plates were not sufficiently sensitive in the red, a new method was adopted in order to bring out the outlines of the first bands.

The whole spectrum was allowed to act upon the plate for 50 sec., a 10 mm. thick layer of a 0.05 per cent, aqueous solution of crocein scarlet was then inserted as a red filter and the plate exposed further. The filter absorbed the light completely up to about $\lambda = 590$ $\mu\mu$. This method proved valuable for completing the picture in the red region even though clearness in presentation was reduced in this way.

In the case of the photographs that were reproduced in the XVIIth paper, the supplementary exposure was not manipulated with sufficient adjustment to the variable relations of the individual photographs. The same duration of supplementary exposure was used for all pigments and for every thickness of layer. The bands, in the case of thin layers, are consequently much weakened upon the plate as a result of over-exposure, especially so in the case of the *b* derivatives in the red, although they are still observable in the spectroscope as dark bands.

Recently this error has been avoided by a shorter supplementary exposure in the case of thin layers so that the spectrographic representation now agrees in all its essential points with the image that is observed in the spectroscope. The difference between corresponding members of the *a* and *b* series as regards the intensity of the chief absorption in the red now approximately reproduces the natural relations; *b* absorbs less strongly in the red than does *a*; in the violet, *a* does not absorb as intensively as *b*.

Our new spectrographic photographs have been reproduced in the appended plates VIII–XI.

In every case, solutions of 0.043 g. of chlorophyll in 1 l. of ether or equimolecular solutions of its derivatives (0.042 g. of pheophytin and 0.030 g. of chlorin *e* or rhodin *g*) were used in thicknesses of 2½, 5, 10, 20 and 40 mm.

The exposure without the use of the red filter lasted 50 seconds. Supplementary exposures were then made with layers of the chlorophyll *a* series as follows: 6 minutes for 2½ mm.; 7 minutes for 5 mm.; 8 minutes for 10 mm.; 9 minutes for 20 mm., and 10 minutes for 40 mm. The chief absorption in the red of the *b* derivatives, when contrasted with that of the *a*, lies somewhat further toward the violet and consequently in a spectral region which acts more strongly upon the plate. They require, therefore, a shorter supplementary exposure; namely, 3 minutes for a 2½ mm. layer, 4 minutes for a 5 mm. layer, 6 for a 10 mm., 8 for a 20 mm. and 10 for a 40 mm. layer.

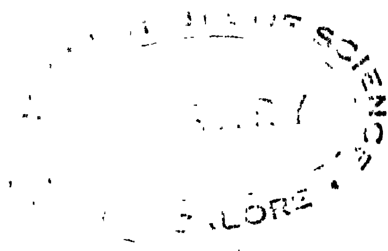
The spectra of the yellow pigments were photographed for 10 mm. thick layers of solutions that contained 0.005 g. of substance in 1 l. of alcohol or carbon disulphide. The time exposure was 75 seconds.

The plates were developed 3 minutes with metol-hydroquinone[4] in complete darkness and then fixed in an acid bath.

Comparison of the photographs and diagrams convinces us that photographic reception and representation of the spectra is not, as is usually assumed, a method possessing greater objectivity than observation with the eye and graphical reproduction of the measurements that are thus obtained. We do not wholly agree, therefore, with the view of Rost, Franz and Heise, who emphasize "the completely objective fixation of pictures" as an undeniable advantage of photography. The

[4] Solution I: 500 cc. of distilled water, 50 g. of crystallized Na_2SO_4, 5 g. hydroquinone and 1 g. metol; Solution II: 500 cc. of distilled water and 50 g. of K_2CO_3. Use 30 cc. of I, 30 cc. of II and 60 cc. of water; temperature 18–20°.

photographic method is also characterized by considerable subjectivity which is conditioned by the sensitiveness of the plate, the illumination, the procedure and by the reproduction. Even though our photograms reproduce all the observed absorption bands correctly according to their position, nevertheless their boundaries and relative intensities are somewhat less exactly shown than in the case of direct observation with the eye, the sensitiveness of which exceeds that of the photographic plate.



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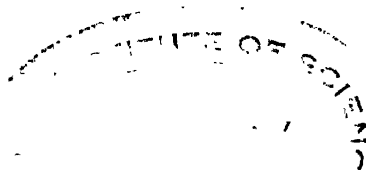
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PLATE II.

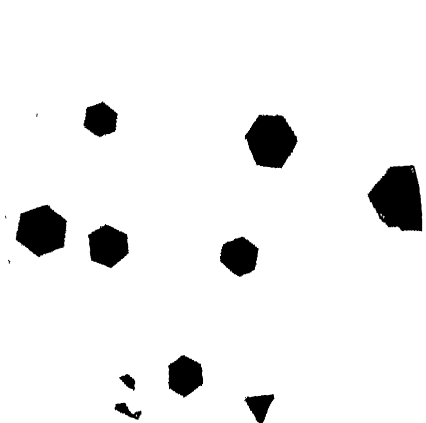


Fig. 1. Crystallized chlorophyll (ethyl chlorophyllide).

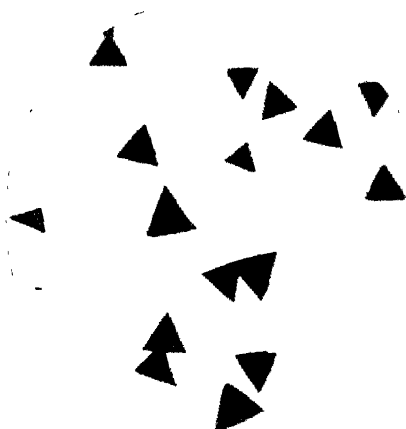


Fig. 2. Ethyl chlorophyllide crystallized from methylal.

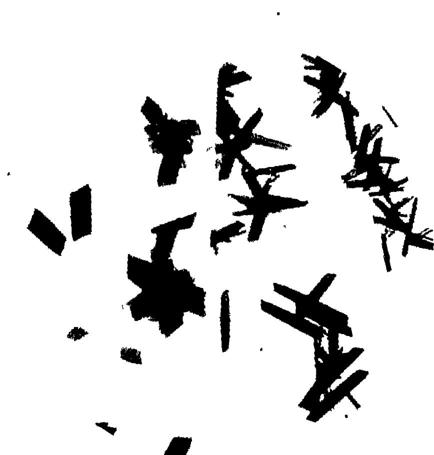


Fig. 3. Methyl chlorophyllide *a*.

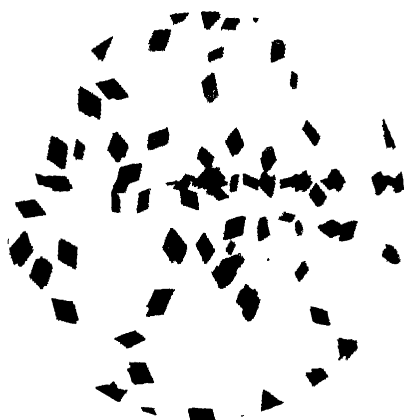


Fig. 4. Methyl chlorophyllide *b*.

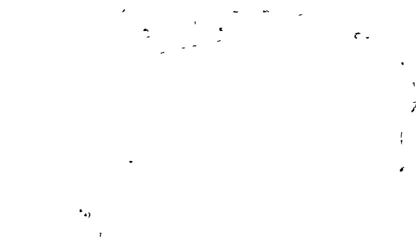






Fig. 1. Carotin crystallized from carbon disulphide-alcohol.

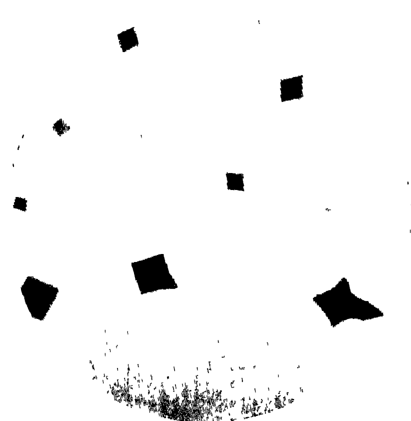


Fig. 2. Carotin crystallized from petroleum ether.



Fig. 3. Xanthophyll crystallized from ethyl alcohol.



Fig. 4. Xanthophyll crystallized from methyl alcohol.

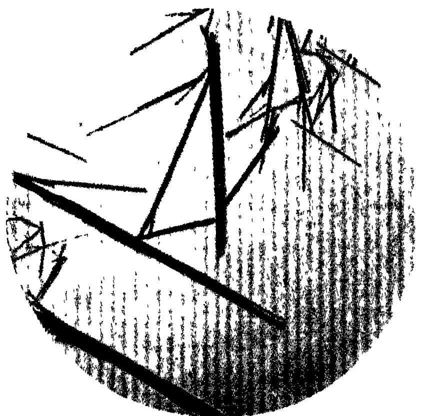




Fig. 6. Fucoxanthin crystallized from



Fig. 1. Methyl pheophorbide *a*.

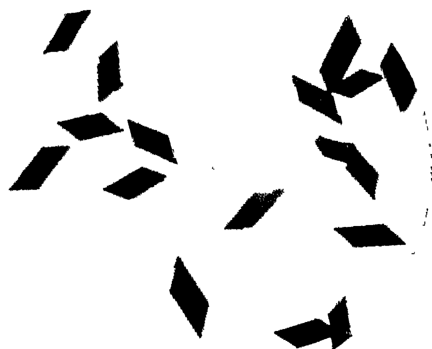


Fig. 2. Methyl pheophorbide *b*.



Fig. 3. Phytochlorin *e*.



Fig. 4. Phytorhodin *g*.



Fig. 5. Phytochlorin *f*.

PLATE V.



Fig. 1. Glaucophyllin.



Fig. 2. Rhodophyllin.



Fig. 3. Pyrrophyllin.

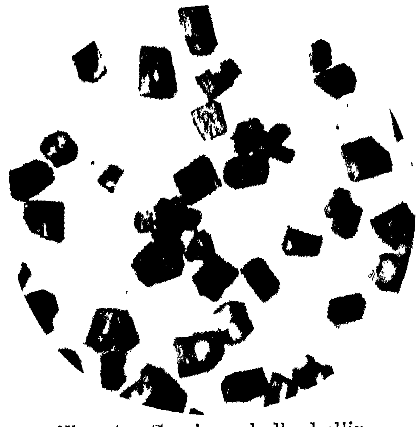


Fig. 4. Caesium-phyllophyllin.



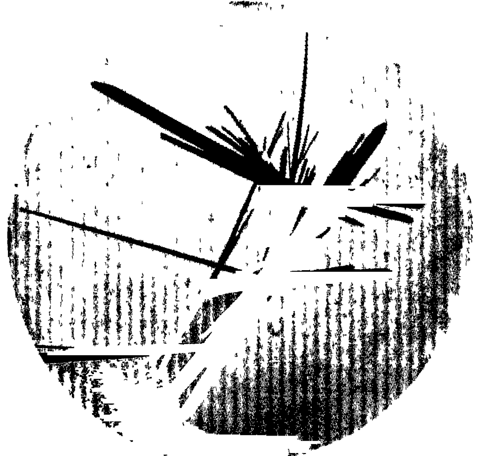
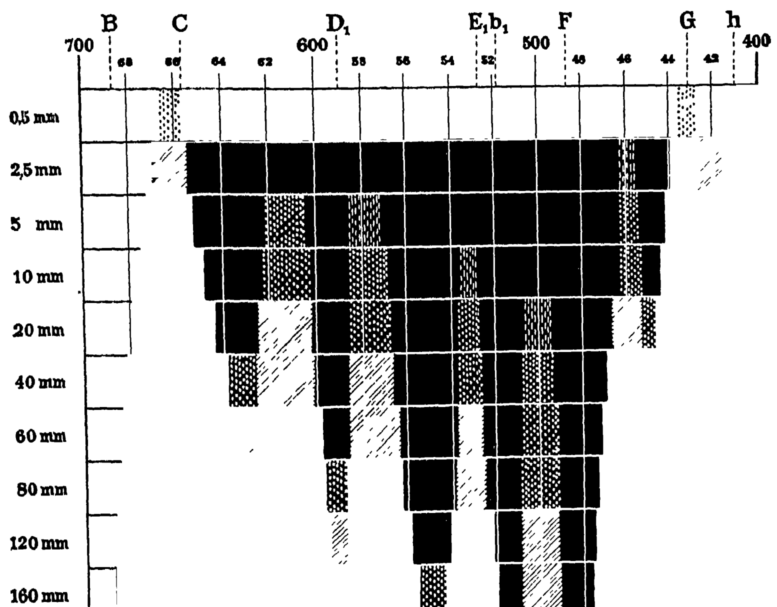


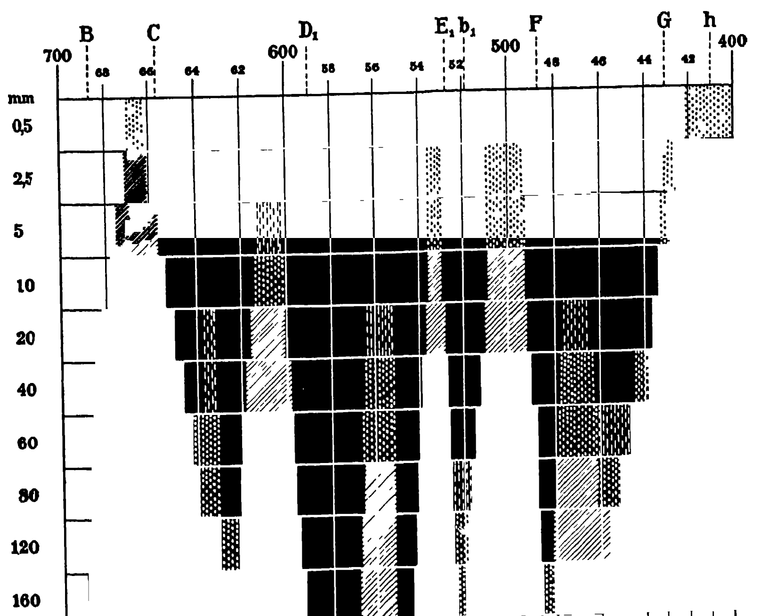


PLATE VI.



The methyl chlorophyllides: *a* (above) and *b* (below).

PLATE VII.



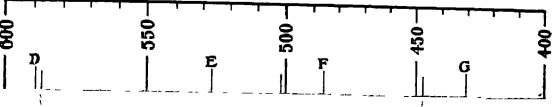
700 B C 600 D1 E1 b1 500 F G h 400

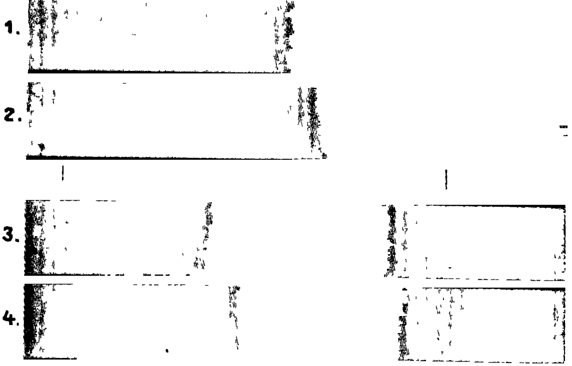
mm
0,5
2,5
5
10
20
40
60
80
120
160

68 66 64 62 600 58 56 54 52 500 48 46 44 42 400
700 B C D1 E. b1 F G h

The methyl pheophorbides: *a* (above) and *b* (below).

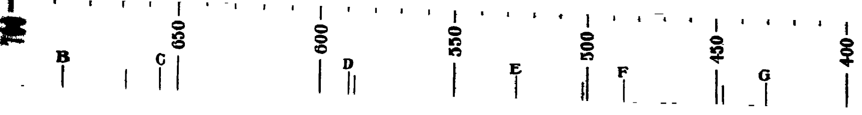
PLATE XI.



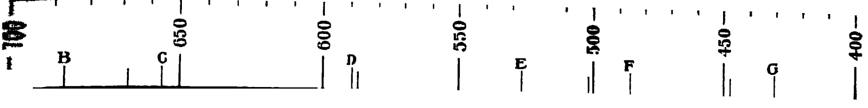


1. Carotin dissolved in alcohol.
2. Xanthophyll dissolved in alcohol.
3. Carotin dissolved in carbon disulphide.
4. Xanthophyll dissolved in carbon disulphide.

PLATE X.

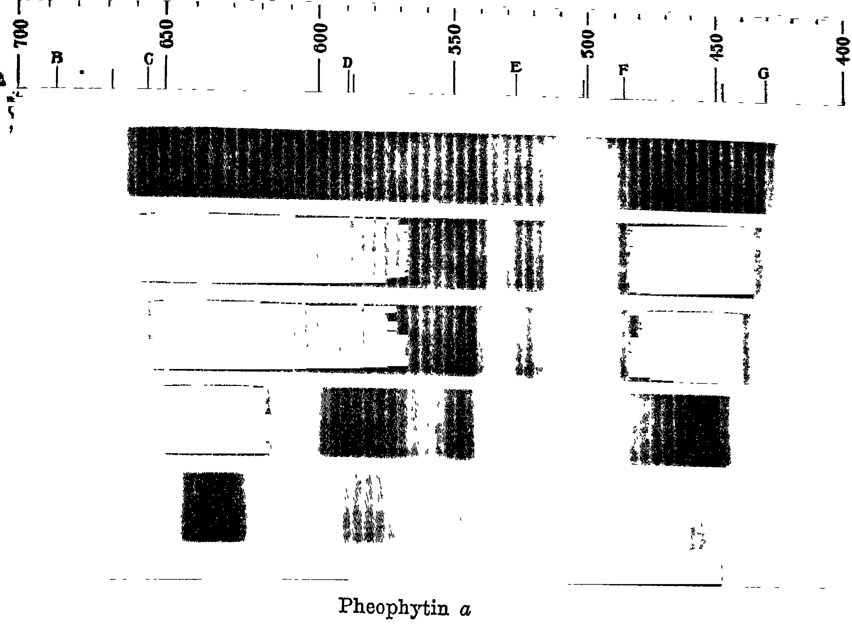


Phytochlorin *e*

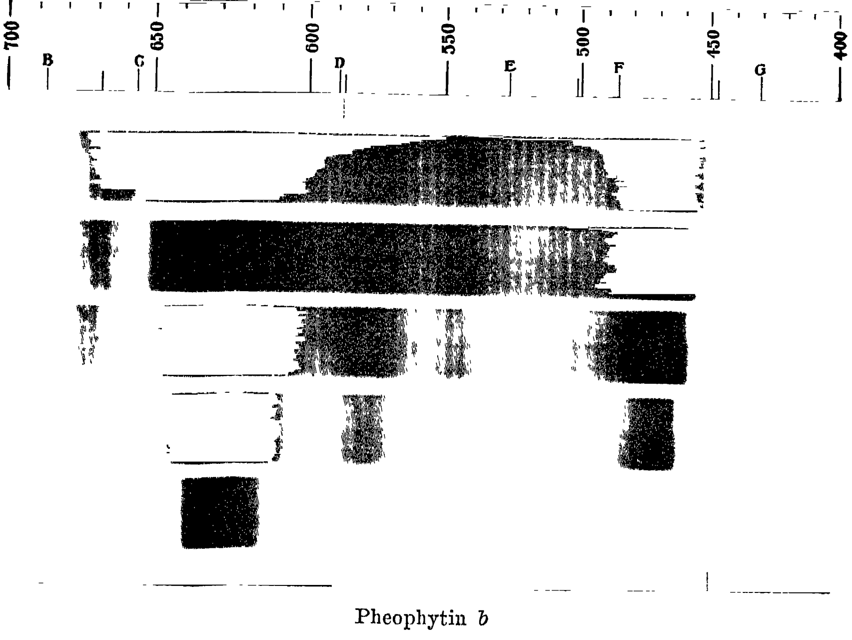


Phytorhodin *g*

PLATE IX.

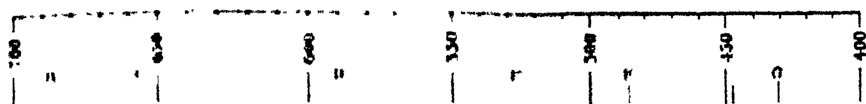


Pheophytin *a*



Pheophytin *b*

PLATE VIII

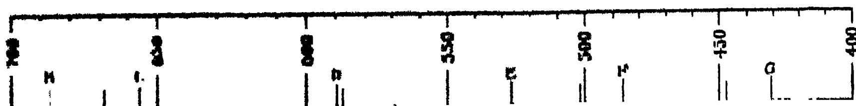


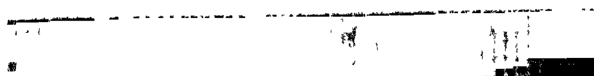







Chlorophyll *a*









Chlorophyll *b*

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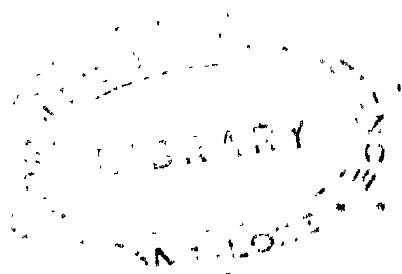

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PLATE I.

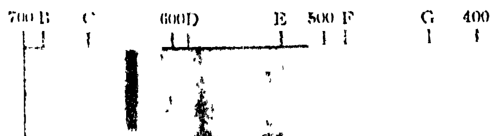


Fig. 1. Spectrum of the leaf pigment, in acetone.

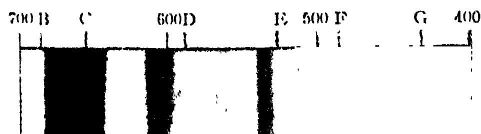


Fig. 2. Spectrum of the acidified extract.

Chlorophyll *a*.

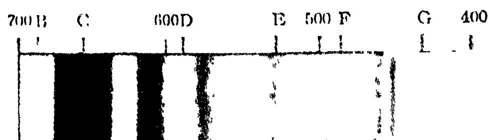
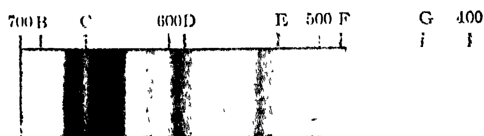


Fig. 3. Spectrum of the two chlorophyll components.



Chlorophyll *b*.

Carotin.

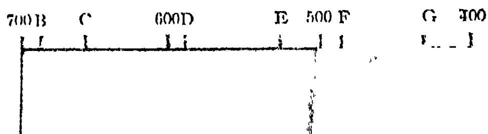
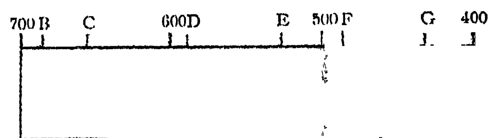


Fig. 4. Spectrum of the yellow pigments.



Xanthophyll.